

**n-3 POLYUNSATURATED FATTY ACIDS SUPPRESS MITOCHONDRIAL
TRANSLOCATION TO THE IMMUNOLOGICAL SYNAPSE AND
MODULATE CALCIUM SIGNALING IN T CELLS**

A Thesis

by

RAJESHWARI YOG

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2010

Major Subject: Biomedical Science

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ABSTRACT

n-3 Polyunsaturated Fatty Acids Suppress Mitochondrial Translocation to the
Immunological Synapse and Modulate Calcium Signaling in T Cells. (December 2010)

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Co-Chairs of Advisory Committee: Dr. Robert S. Chapkin
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T helper (Th) cell activation is necessary for the adaptive immune response. Formation of an immunological synapse (IS) between Th cells and antigen-presenting cells is the first step in Th cell activation. In vitro studies indicate that formation of the IS induces cytoskeleton-dependent mitochondrial redistribution to the immediate vicinity of the IS. This redistribution of mitochondria to the IS in T cells is necessary to maintain Ca^{2+} influx across the plasma membrane and Ca^{2+} -dependent Th cell activation. Earlier studies have demonstrated that n-3 polyunsaturated fatty acids (PUFA) suppress the localization and activation of signaling proteins at the IS. Therefore, we hypothesized that n-3 PUFA suppress CD4^+ T cell mitochondrial translocation during the early stages of IS formation and down-modulate Ca^{2+} dependent Th cell activation. CD4^+ cells derived from *fat-1* mice, a transgenic model that synthesizes n-3 PUFA from n-6 PUFA, were co-cultured with anti-CD3-expressing hybridoma cells (145-2C11) for 15 min at 37°C, and mitochondrial translocation to the IS was assessed by confocal microscopy. *fat-1* mice exhibited a significantly ($P < 0.05$) reduced percentage of CD4^+ T cells with mitochondria which translocated to the IS; *fat-1* (30%) versus wild type

control (82%). With respect to an effect on the mitochondrial-to-cytosolic Ca^{2+} ratio, wild type cells showed significant increases at the IS (71%) and total cell (60%) within 30 min of IS formation. In contrast, *fat-1* CD4^{+} T cells remained at basal levels following the IS formation. A similar blunting of the mitochondrial-to-cytosolic Ca^{2+} ratio was observed in wild type cells co-incubated with inhibitors of the mitochondrial uniporter, RU360 or calcium release-activated Ca^{2+} (CRAC) channels, BTP2. Together, these observations provide evidence that n-3 PUFA modulate Th cell activation by limiting mitochondrial translocation to the IS and reducing Ca^{2+} entry.

DEDICATION

To

My mother, Dr. (Maj.) Indira Yog for her strength to take on whatever life throws at her
with a smile; my teachers and friends who have been with me every step of the way,
through good times and bad. Thank you for everything.

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NOMENCLATURE

Ab	Antibody
APC	Antigen-presenting cells
ATCC	American type culture collection
Ca ²⁺	Calcium
CD-3	Cluster of differentiation 3
CD4 ⁺	Cluster of differentiation 4
CRAC	Ca ²⁺ release Ca ²⁺ activated channels
CRAC/ORAI1	Calcium release-activated calcium channel encoded by the ORAI1 gene
DHA	Docosahexaenoic acid
DNP	Dinitrophenol
EPA	Eicosapentaenoic acid
FBS	Fetal bovine serum
IL-2	Interleukin-2
IS	Immunological synapse
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
NFAT	Nuclear factor activated T cell
NKC	Natural killer cells
PKC	Protein kinase C

PLC	Phospholipase C
PUFA	Polyunsaturated fatty acids
ROI	Region of interest
TCR	T cell receptor
Th1	T-helper 1
TIFF	Tagged image file format
WT	C57BL/6 wild type

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW*

T cell activation requires contact with antigen-presenting cells (APCs) to bring the T cell receptor (TCR) and major histocompatibility complex (MHC)-peptide complex together. In general, contact is defined by the size of the TCR and MHC-peptide complex which, at approximately 10-15 nm, requires extensive interdigitation of the plasma membrane of the T cell and APC. T cells are typically activated via formation of a stable T cell-APC junction, referred to as an immunological synapse (IS)³ (1-4).

When the T cell receptor is activated by antigen presentation, mitochondria redistribute close to the site of the IS, where they promote influx of Ca^{2+} (5). The actin cytoskeleton-based recruitment of mitochondria in the vicinity of active synapses is required for Ca^{2+} influx through calcium release-activated calcium (CRAC) channels and activation of key downstream transcription factors such as NFAT. The translocation of the mitochondria to the IS is typically observed within 15 min after focal TCR cross-linking (5) with a sustained peak around 30 min (6). Reduction in the T cell Ca^{2+} concentration close to the channel serves as a feedback inhibitory mechanism responsible for adjusting channel activity (5). Any agent which inhibits the movement of

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mitochondria to the IS and modulates intracellular Ca^{2+} homeostasis can, therefore, prevent sustained IS formation, thereby suppressing T cell activation and function. A plethora of published literature supports the contention that dietary omega-3 polyunsaturated fatty acids (PUFA), eicosapentaenoic (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) in particular, are important modulators of a host's inflammatory/immune responses (7-9).

These studies demonstrate that EPA and DHA reduce pro-inflammatory responses, in part, by diminishing T cell proliferative capacity in response to both mitogenic and antigenic stimulation (10). Previous studies have demonstrated that n-3 PUFA modulate CD4^{+} T cell immune response via reduction in T-helper 1 (Th1) effector cell clonal expansion mediated, in part, by diminished interleukin-2 (IL-2) secretion and IL-2R α -chain mRNA transcription (11, 12). However, the molecular mechanisms by which n-3 PUFA suppress CD4^{+} T cell function are not fully understood. Recently, it was demonstrated that n-3 PUFA are capable of suppressing the localization and activation of signaling proteins at the IS in mouse T cells (12, 13).

Mammals cannot produce n-3 PUFA from the major n-6 PUFA found in the diet due to lack of $\Delta 15$ -desaturase activity. Hence, it is necessary to enrich the diet with EPA and/or DHA to assess their biological properties in vivo. However, transgenic mice expressing the *fat-1* gene encoding an n-3 fatty acid desaturase cloned from *Caenorhabditis elegans* can catalyze the conversion of n-6 PUFA to n-3 PUFA by introducing a double bond into fatty acyl chains (14). Therefore, the *fat-1* mouse model

facilitates the investigation of the biological properties of n-3 PUFA in T cells without having to incorporate these fatty acids into the diet.

In this study, the effect of n-3 PUFA on T cell-APC interactions in *fat-1* mice was investigated. Specifically, the effect of n-3 PUFA on the recruitment of mitochondria to the IS during the initial activation phase of T cells in contact with APCs and the consequent changes in Ca^{2+} signaling in T lymphocytes were investigated. We demonstrate for the first time that n-3 PUFA modulate the early Ca^{2+} mediated events following the interaction of T cell with APCs. These results are consistent with the ability of n-3 PUFA to inhibit downstream signaling and suppress T cell activation.

1.1 Hypotheses

Using the *fat-1* mouse model, the effect of n-3 PUFA on mitochondrial translocation and Ca^{2+} uptake at the T cell immunological synapse were investigated. Based upon earlier documentation of suppression of localization and activation of signaling proteins at the IS by dietary EPA and DHA, it was hypothesized that n-3 PUFA enrichment of T cell membranes of *fat-1* mice will (**Figure 1**);

- 1) Suppress the localization of mitochondria at the IS of CD4^{+} T cells,
- 2) Inhibit Ca^{2+} uptake by mitochondria at the IS, and
- 3) Decrease the mitochondrial-to-cytosolic Ca^{2+} ratio.

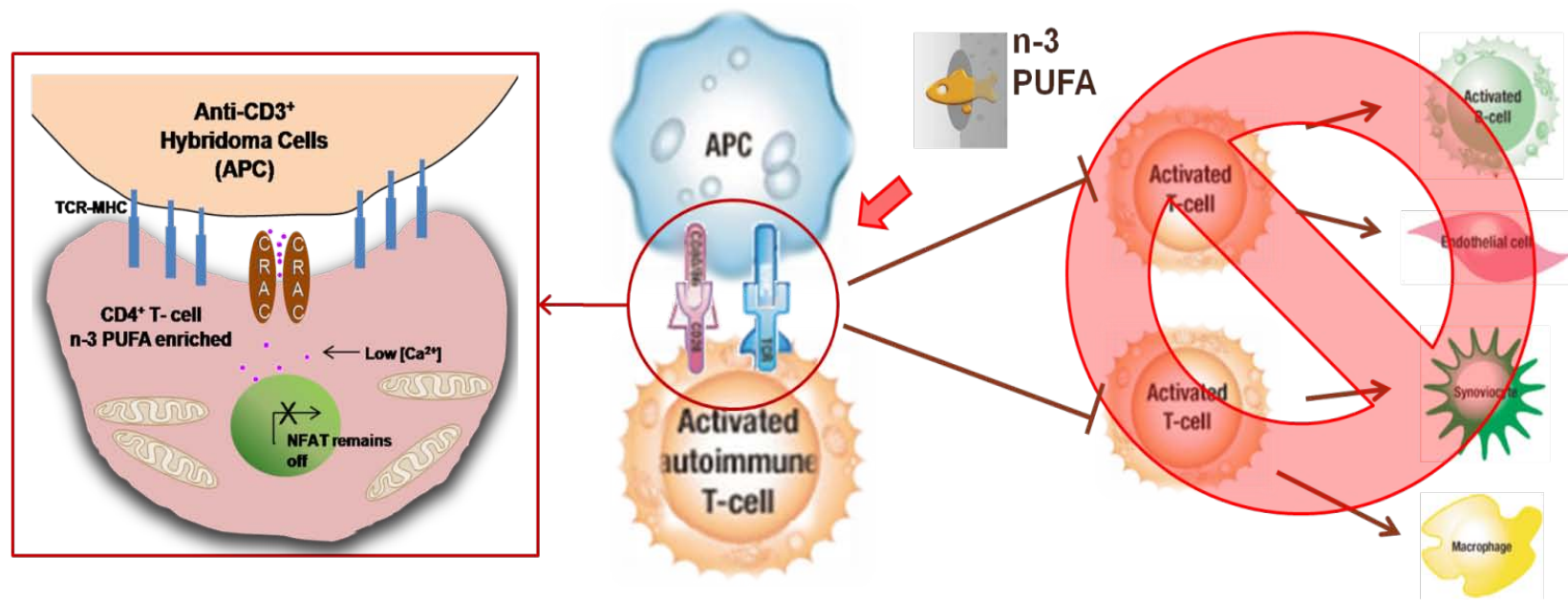


Figure 1. n-3 PUFA inhibits mitochondrial translocation and suppresses Ca²⁺-dependent T cell activation.

1.2 Experimental approach

1.2.1 Specific aim 1

Determine if n-3 PUFA inhibit mitochondrial translocation to the vicinity of the immunological synapse.

To investigate the effect of endogenous n-3 PUFA on mitochondria translocation to the immunological synapse, Mitotracker green labeled CD4⁺ T cells from *fat-1* and WT mice were cocultured with anti-CD-3 hybridoma cells at 37°C in 5% CO₂ for 0-30 min. Images of conjugate CD4⁺ T cells and hybridoma cells were captured, whereas non-contact CD4⁺ T cells serve as negative controls. An assessment of mitochondrial translocation to the IS over time was assessed. The DNP –specific Ab expressing hybridoma were also used as a negative control.

1.2.2 Specific aim 2

Determine if n-3 PUFA reduce Ca²⁺ uptake by mitochondria at the immunological synapse.

Mitochondrial modulation of CRAC channel activity is involved in mediating the translocation of mitochondria toward the IS at the plasma membrane (5). Translocation to the IS allows mitochondria to take up a large amount of incoming Ca²⁺ directly beneath the mouth of the channels, thereby reducing channel inactivation (5). Therefore, the effect of n-3 PUFA on mitochondrial Ca²⁺ levels using Mitotracker Green and Rhod-2 AM probes in splenic CD4⁺ T-cells from *fat-1* and WT mice were investigated.

1.2.3 Specific aim 3

Determine if n-3 PUFA modulates intracellular calcium signaling by altering the mitochondrial-to-cytosolic Ca^{2+} ratio.

To further document the effects of n-3 PUFA on intracellular Ca^{2+} signaling in T cells, Fluo-4, a visible wavelength non-ratiometric cytosolic indicator that exhibits a 40-fold increase in fluorescence intensity with Ca^{2+} binding (7), was loaded along with Rhod-2 AM (2.5 μM) in order to evaluate the mitochondria-to-cytosolic Ca^{2+} ratio. The relative fluorescence intensities of Rhod-2 to Fluo-4 was measured in order to determine the mitochondrial-to-cytosolic ratio in living CD4^+ T cells from *fat-1* and WT mice. To further evaluate the role of mitochondrial Ca^{2+} uptake, cells were pre-incubated with 10 μM RU-360, an inhibitor of mitochondrial uniporter, for 30 min prior to coculture with hybridoma cells. Cells were then co-loaded with fluorescent Ca^{2+} indicators and the ratio of cytosolic to mitochondrial Ca^{2+} was measured as described in materials and methods section. To examine the influx of Ca^{2+} from the extracellular environment through the calcium dependent membrane channel; in separate experiments; cells were incubated with 10 μM BTP2, a pharmacological inhibitor of CRAC/ORAI1 channel activity, for 1 h prior to the calcium labeling with Rhod-2 and Fluo-4, subsequently mitochondrial-to-cytosolic Ca^{2+} ratio was determined.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

RPMI 1640 medium and sodium pyruvate were purchased from Cellgro (Manassas, VA). Fetal bovine serum (FBS) was purchased from Irvine Scientific (Santa Ana, CA). DMEM 1X medium, Leibovitz medium, Penicillin, Streptomycin and Glutamax were purchased from Gibco (Carlsbad, CA). Fluo-4 AM, Rhod-2 AM, and MitoTracker Green FM were purchased from Molecular Probes (Eugene, OR). RU-360 and BTP2 were purchased from Calbiochem (San Diego, CA). Two-well Lab-Tek chambered cover glass slides were purchased from Nunc (Naperville, IL). Poly-L-lysine solution was purchased from Sigma-Aldrich (St. Louis, MO). Stock solutions of mitotracker green, Fluo-4 AM, Rhod-2 AM and BTP2 were prepared in DMSO and diluted with medium to 100 nM, 1.0 μ M, 1.5 μ M, and 10 μ M, respectively (final concentration of the vehicle DMSO was maintained at 0.1–0.3% in culture). Stock solutions of RU-360 was prepared in degassed water and diluted with medium to a final concentration of 10 μ M.

2.2 Cell culture

Hybridoma cells expressing hamster mAbs specific for either murine CD3 (clone 145-2C11) or an irrelevant antigen, DNP (clone UC8-1B9, anti-DNP hybridoma) were obtained from American Type Culture Collection (Manassas, VA). Cells were

maintained in DMEM-1X with 10% FBS, 4 mM L-glutamine, 1 mM sodium pyruvate, nonessential amino acids, and 10^5 U/L penicillin and 100 mg/L streptomycin at 37°C and 5% CO₂. In addition to the presence of CD3 engagement, both types of hybridoma also display significant surface levels of the co-stimulatory ligands B7 and ICAM-1, which are capable of engaging their counterparts on the T cell (15). This model has been extensively used to examine T cell/APC interaction (16).

2.3 Animals and CD4⁺ T cell isolation

Fat-1 transgenic mice were generated and backcrossed onto a C57BL/6 background as previously described (14, 17). All procedures followed guidelines approved by Public Health Service and the Institutional Animal Care and Use Committee at Texas A&M University. The colony of *fat-1* mice used for this study was generated by breeding heterozygous mice. Mice were genotyped using tail DNA. To confirm the phenotype, total lipids were isolated from splenic T-cells and the fatty acid profile was characterized by gas chromatography as previously described (17, 18). Specific pathogen-free animals were maintained under barrier conditions and were fed a 10% safflower oil diet (n-6 PUFA rich, Research Diets) *ad libitum* with a 12 h light/dark cycle. The diet contained 40 (g/100 g diet) sucrose, 20 casein, 15 corn starch, 0.3 DL-methionine, 3.5 AIN 76A salt mix, 1.0 AIN 76A mineral mix, 0.2 choline chloride, 5 fiber (cellulose), 10 safflower oil. CD4⁺ T cells from *fat-1* or wild type mice were isolated from spleens by a magnetic microbead positive selection method (13).

2.4 CD4⁺ T cell mitochondria labeling using Mitotracker Green FM (mitochondrial localization probe)

Purified (>92.0 % determined by flow cytometry) CD4⁺ T cells (2 x 10⁶ cells/ml) were labeled with Mitotracker Green FM for 15 min at 37°C in RPMI 1640 (complete medium with 10% FBS). Mitotracker Green is a green-fluorescent mitochondrial stain that localizes in the mitochondria regardless of membrane potential (19) (**Figure 2**). This probe is essentially nonfluorescent in aqueous solution, only becoming fluorescent when it accumulates in the lipid environment of the mitochondria.

2.5 CD4⁺ T cell mitochondria Ca²⁺ labeling using Rhod-2 AM (mitochondrial Ca²⁺ indicator)

Isolated CD4⁺ T cells (2 x 10⁶ cells/ml) were labeled with red fluorescent dye, Rhod-2 AM for 30 min at 37°C. Cells were subsequently washed to remove cytosolic Rhod-2 and resuspended in serum-free Leibovitz medium. Mitotracker Green FM was used to confirm that Rhod-2 fluorescence corresponded to mitochondrial Ca²⁺ accumulation (19) (**Figure 2**).

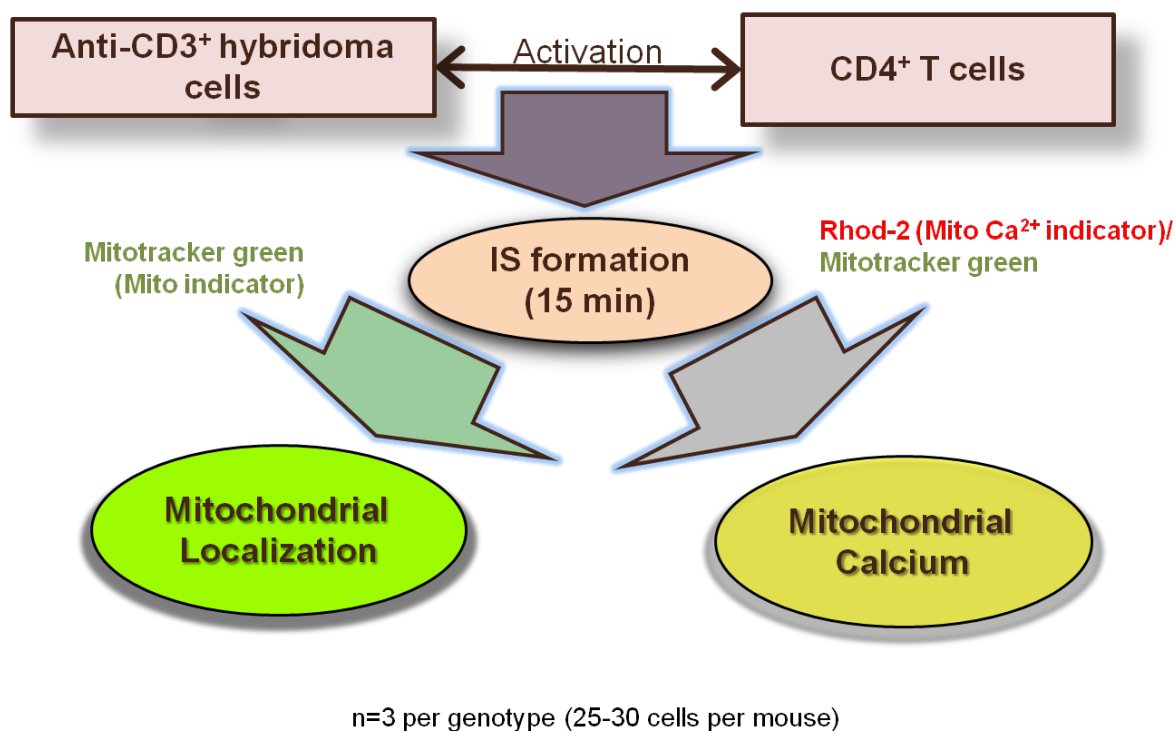


Figure 2. Experimental design to investigate the effect of n-3 PUFA on mitochondrial translocation and Mitochondrial Ca²⁺.

2.6 CD4⁺ T cell mitochondrial-to-cytosolic calcium labeling using Rhod-2 AM and Fluo-4 (cytosolic Ca²⁺ indicator)

Isolated CD4⁺ T cells (2 x 10⁶ cells/ml) were incubated with green fluorescent dye, Fluo-4, and red fluorescent dye, Rhod-2, for 1 h at 37°C. Fluo-4 is a visible wavelength non-ratiometric cytosolic Ca²⁺ indicator that exhibits a 40-fold increase in fluorescence intensity with Ca²⁺ binding (20). Following 1 h incubation with the probe, cells were washed with Leibovitz medium and imaged. The ratio of the cytosolic-to-mitochondrial Ca²⁺ level was subsequently calculated. In addition, experiments were performed in cultures incubated with RU-360, an inhibitor of mitochondrial uniporter, for 30 min prior to the calcium labeling with Rhod-2 and Fluo-4, following which the

mitochondrial-to-cytosolic Ca^{2+} ratio was determined. In separate experiments, cells were incubated with BTP2, a pharmacological inhibitor of CRAC/ORAI1 channel activity (21, 22), for 1 h prior to the calcium labeling with Rhod-2 and Fluo-4, after which the mitochondrial-to-cytosolic Ca^{2+} ratio was determined. The mitochondrial-to-cytosolic Ca^{2+} ratio represents the relative fluorescence intensities of Rhod-2 and Fluo-4.

2.7 CD4⁺ T-cell activation and fluorescence microscopy

For mitogenic CD4⁺ T cell activation, an anti-CD3 expressing hybridoma (clone 145-2C11, ATCC) was used. Mitotracker / Rhod 2 labeled CD4⁺ T cells were co-incubated with hybridoma cells at a 5:1 ratio. Cell mixtures were seeded onto poly-L-lysine pre-coated Lab-tek two-well chambered coverglass slides (2×10^6 CD4⁺ T-cells/chamber). Cell co-cultures were incubated for 15 min at 37°C in 5% CO₂. For quantification of Rhod-2 and Fluo-4 fluorescence, excitation wavelengths of 488 and 550 nm were used and fluorescence emission was monitored at 530 and 590 nm, respectively. Ten-15 areas with 20–25 cells per area and 4–8 wells per treatment were imaged with a 510 META NLO laser scanning microscope (Carl Zeiss Microimaging, Thornwood, NY) equipped with an argon laser and LSM software. Images were collected using a 63X objective (1.3 numerical aperture). To validate IS formation, T cells were incubated with irrelevant DNP-specific Ab expressing hybridoma cells (clone UC8-1B9, anti-DNP hybridoma) as a negative control (13, 16).

2.8 Image processing and quantification of mitochondrial-to-cytosolic Ca^{2+}

All images were acquired by confocal microscopy and were converted to 8-bit/channel TIFF format, and the percentage of cells with mitochondrial translocation to the IS was determined. To assess mitochondria-to-cytosolic calcium at the synapse and whole cell, polygons were drawn to designate the IS and the contact whole cell area. The normalized Rhod-2 intensity, an indicator of mitochondrial calcium, and normalized Fluo-4 intensity, an indicator of cytosolic calcium, was calculated by using mean fluorescence intensity (MFI) according to the formula: (Rhod-2 MFI / Fluo-4 MFI at the synapse and contact whole cell). Calcium measurements were determined in individual cells for each experiment, and comparisons were made with same day controls, including inhibitors. Quantitative analysis of MFI was performed using Adobe Photoshop CS3 for Windows.

2.8.1 Statistical analysis

The effect of the independent variable (*fat-1* vs wild type) was assessed using SPSS 15.0 for Windows. Data are expressed as mean \pm SE. More than three independent experiments were performed for each experimental condition. Differences between means were tested using t/F- type tests of contrast (two-tailed). P values <0.05 were considered to be statistically significant.

CHAPTER III

RESULTS

3.1 n-3 PUFA inhibit mitochondrial translocation to the vicinity of the immunological synapse

Both *fat-1* transgenic and wild type control offspring were fed a 10% safflower diet enriched in n-6 PUFA throughout the duration of the study. To investigate the effect of endogenous n-3 PUFA on mitochondria translocation to the immunological synapse, Mitotracker green labeled CD4⁺ T cells were cocultured with anti-CD-3 hybridoma cells at 37°C in 5% CO₂ for 0-30 min. Images of conjugate CD4⁺ T cells and hybridoma cells were captured, whereas non-contact CD4⁺ T cells served as negative controls. The DNP-specific Ab expressing hybridoma was used as a negative control, and failed to exhibit direct mitochondrial translocation to the immediate vicinity of the IS formation (**Figures 3A - C**). Typically, greater than 75% of wild type (control) CD4⁺ cells exhibited a directed movement of mitochondria to the IS by 15 min (**Figures 3C & D**). In contrast, CD4⁺ T cells from *fat-1* mice had a significantly ($p < 0.05$) reduced percentage of cells with mitochondria translocation to the IS (**Figure 4**). Typically, mitochondria in T cells are localized preferentially in one area of the cell (5). This apparent polarized mitochondrial localization may be caused by the rather large nucleus in T cells, and explains why a “localized” mitochondrial distribution was observed (**Figure 3B**).

In independent experiments in our laboratory, total lipid fatty acid compositional analyses of the *fat-1* CD4⁺ T cell revealed an increase in n-3 PUFA, EPA (20:5 n-3), docosapentaenoic acid (22:5 n-3) and DHA (22:6 n-3), and decrease in n-6 PUFA, arachidonic acid (20:4n-6), adrenic acid (22:4n-6), and docosapentaenoic acid (22:5n-6) (13). In addition, the ratio of n-6 to n-3 PUFA was significantly ($p < 0.05$) suppressed in *fat-1* compared with wild type cells. This indicates that an appropriate activity of n-3 fatty acid desaturase was present and that T cells from *fat-1* mice were enriched in n-3 PUFA. These data demonstrate that n-3 PUFA suppress mitochondrial localization to the IS.

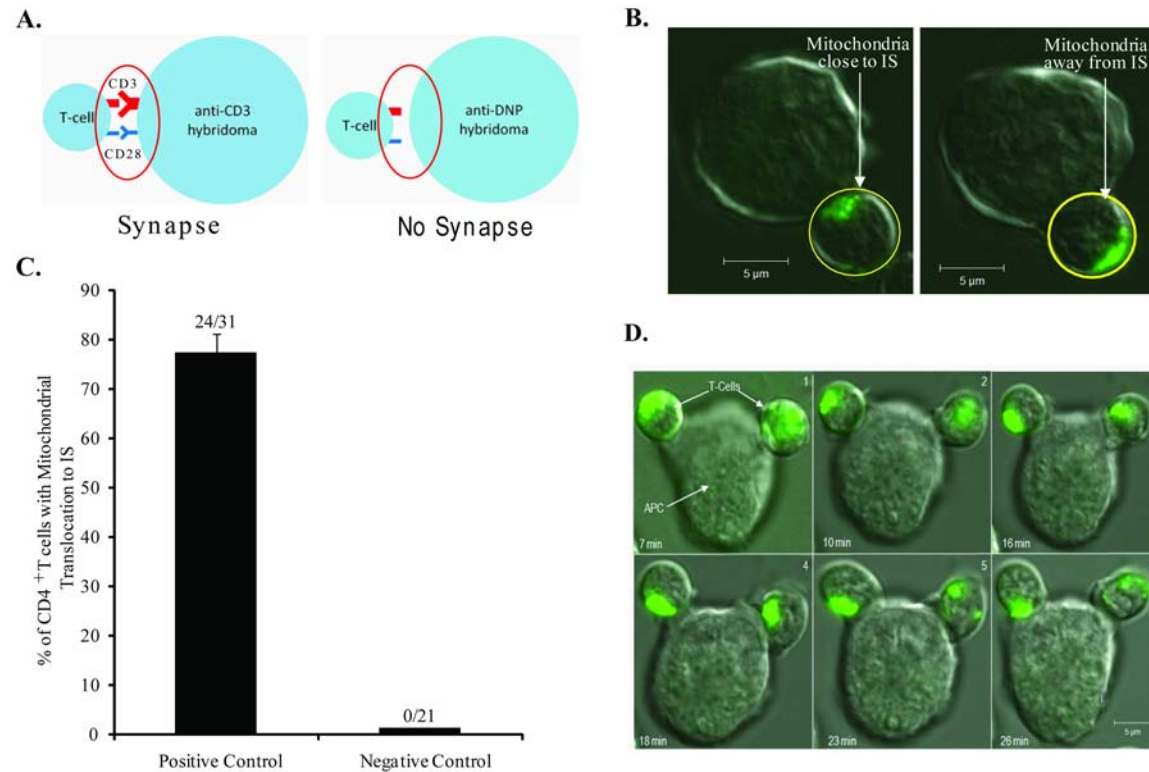


Figure 3. Focal stimulation of T cell receptor activates mitochondrial translocation to the immunological synapse (IS). (A) Schematic of immunological synapse (positive control) formation (left) and non-contact (negative control) immunological synapse (right). (B) Immunofluorescence images from MitoTracker Green/AM loaded CD4⁺ T-cells co-cultured with (left) anti CD3 (positive control) and (right) anti-DNP (negative control) hybridoma. Scale bar represents 5 μ m. (C) Summary image analysis of subplasma membrane localization of mitochondria in wild type CD4⁺ T cells after 15 min co-stimulation with anti-CD3 expressing Hybridoma 145-2C11 (positive control) and irrelevant expressing Ab Hybridoma UC8-1B9 (negative control), mean \pm SEM of n=21-31 cells. (D) Confocal fluorescence images from a single Mitotracker Green/AM- loaded CD4⁺ T cell forming an immunological synapse with an antigen-presenting cell (APC). Image kinetics were measured from time 0 to 30 min following co-incubation of Mitotracker loaded (bright green) CD4⁺ T cells (from a wild type/control mouse) with APC (Hybridoma 145-2C11). Images in each panel represent the same co-incubated cells (7-26 min). Green patching represents mitochondria location in the T cell. Scale bar represents 5 μ m.

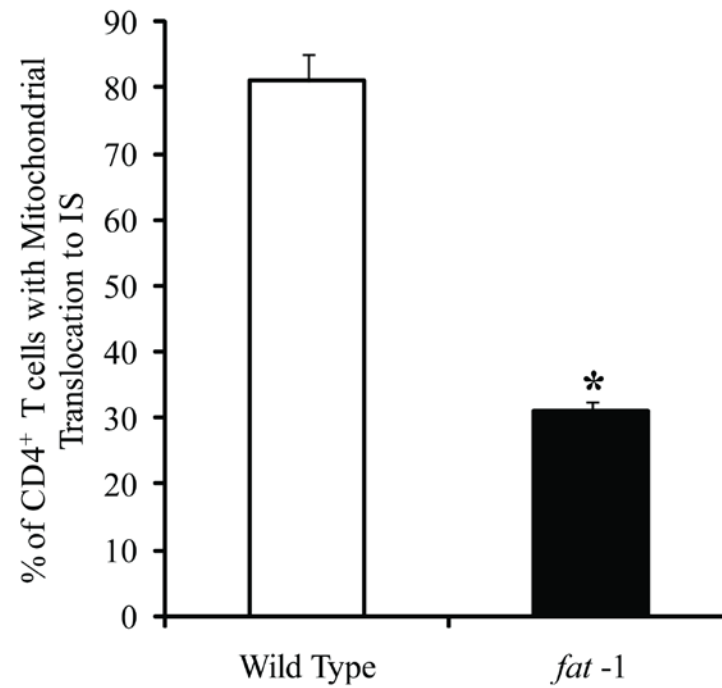


Figure 4. n-3 PUFA suppress mitochondria translocation to the immunological synapse. Subplasma membrane localization of mitochondria to the immunological synapse was quantified in CD4⁺ T cells (n=140 cells from 5 *fat-1* mice) after a 15 min co-stimulation with Hybridoma 145-2C11 antigen-presenting cells. Percentage of cells exhibiting mitochondrial translocation, *fat-1* vs wild type (n=102 cells from 5 wild type mice) comparison. Asterisk indicates significant difference at p<0.05.

3.2 n-3 PUFA reduce Ca^{2+} uptake by mitochondria at the IS

Mitochondrial modulation of CRAC channel activity is involved in mediating the translocation of mitochondria toward the IS at the plasma membrane. Translocation to the IS allows mitochondria to take up a large amount of extracellular Ca^{2+} directly beneath the mouth of the channels, thereby reducing channel inactivation (5). Therefore, we examined the effect of n-3 PUFA on mitochondrial Ca^{2+} levels using Mitotracker Green and Rhod-2 AM probes. In this double-labeling experiment, Rhod-2 at the synapse is an indicator of mitochondrial Ca^{2+} because of the much higher colocalization of both dyes. **Figure 5A** shows representative images documenting differential intracellular localization of Ca^{2+} in mitochondria (yellow pixels). In addition, mitochondrial Ca^{2+} was measured within the region of interest near the IS (**Figure 5B**). In cells exhibiting mitochondrial translocation to the IS, no significant differences in Ca^{2+} concentration between the wild type and *fat-1* T cells was observed (**Figure 5C**). In contrast, there was a significant reduction in Ca^{2+} levels ($p < 0.05$) at the IS in *fat-1* T cells when both translocated and non-translocated mitochondria data were combined (**Figure 5D**). These results indicate that T cell Ca^{2+} signaling at the IS is suppressed in CD4^+ T cells enriched with n-3 PUFA. The higher intramitochondrial Ca^{2+} at the IS is probably caused by the intimate contact between mitochondria and the IS, which exposes mitochondria to higher microenvironments of Ca^{2+} .

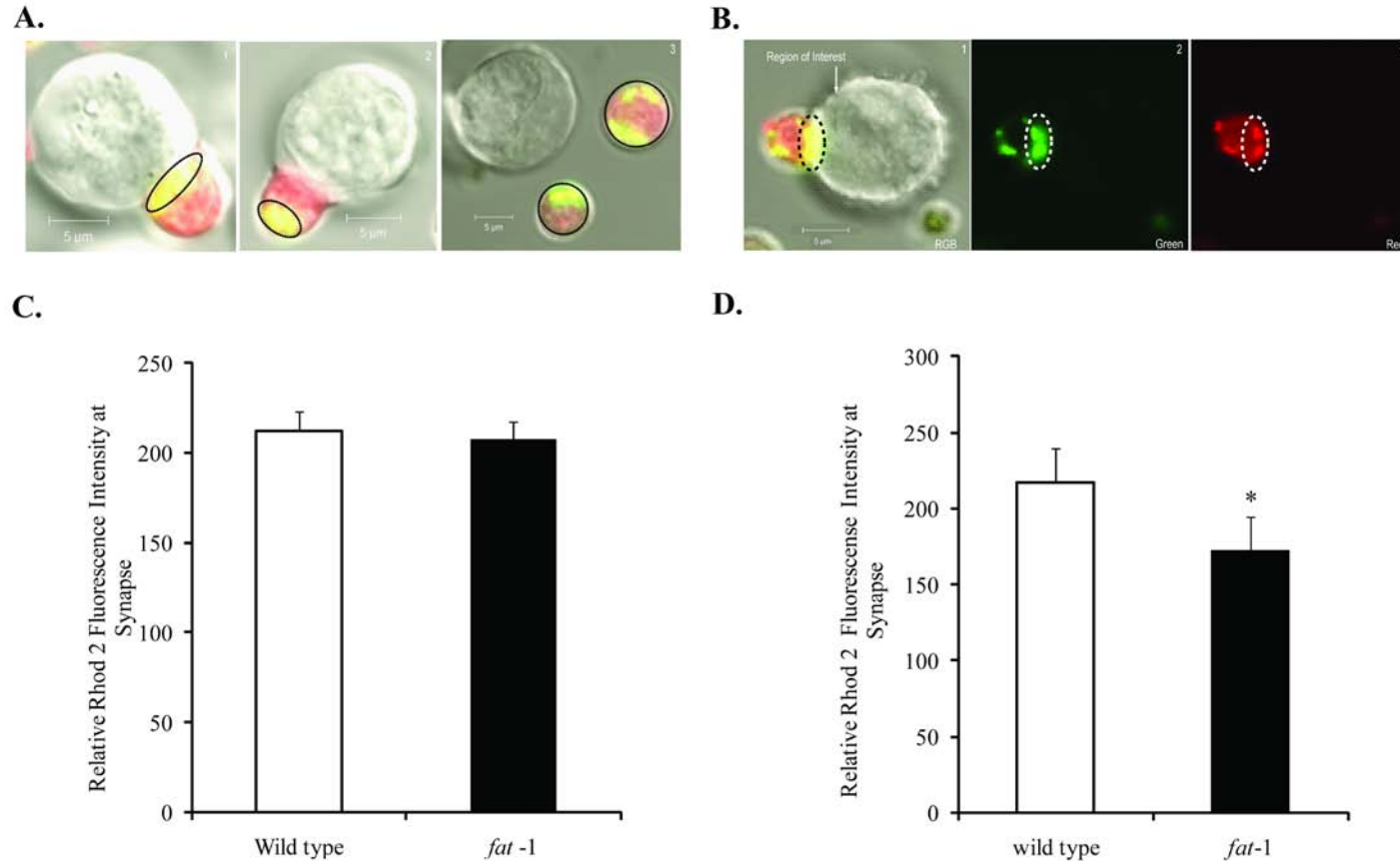


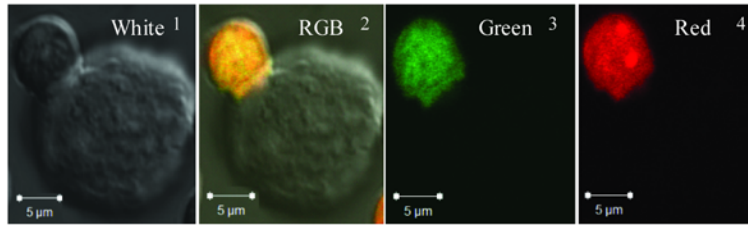
Figure 5. Measurement of Ca^{2+} uptake at the immunological synapse. (A) Shows the region of interest where calcium signal is observed. (A1) T cell with mitochondrial translocation to the IS. (A2) cell exhibiting a failure to translocate mitochondria to the IS, and (A3) T cell with no contact, therefore no IS is formed. (B) Representative confocal microscopy images of Rhod-2 and Mitotracker labeled cells (B1) RGB, (B2) green, (B3) red channels, respectively. Regions of interest (ROI) at the T cell immune synapse were selected by drawing an oval or polygon as shown in dotted lines. Mean intensities from red and green channels were recorded to calculate Ca^{2+} values as described in the Methods. Scale bar, 5 μ m. (C) Effect of n-3 PUFA on Mitochondrial Ca^{2+} at the immunological synapse only in cells with mitochondrial translocation to the IS. (D) Comparison of mitochondrial Ca^{2+} measurements in CD4^{+} T cells in wild type (n=26 cells 3 mice) and *fat-1* (n=18 cells from 3 mice). Asterisk indicates significant difference at $p < 0.05$.

3.3 n-3 PUFA modulate intracellular calcium signaling by altering the mitochondrial-to-cytosolic Ca^{2+} ratio

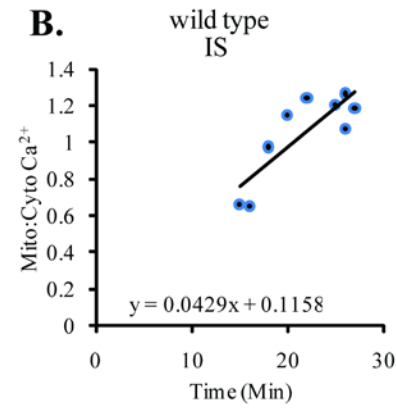
Since mitochondria are key organelles involved in the regulation of intracellular Ca^{2+} homeostasis, the ratio of mitochondrial-to-cytosolic Ca^{2+} in individual cells was measured. Fluo-4, a visible wavelength non-ratiometric cytosolic indicator, was loaded along with Rhod-2 AM in order to evaluate the mitochondrial-to-cytosolic Ca^{2+} ratio. The relative fluorescence intensities of Rhod-2 to Fluo-4 were measured to determine the mitochondrial-to-cytosolic ratio in living CD4^+ T cells from *fat-1* and wild type mice. Since IS formation, mitochondrial translocation, and Ca^{2+} influx peaks by approximately 30 min (23), mitochondrial-to-cytosolic ratio data were collected over a 15-30 min time interval. **Figure 6A** shows representative images of Fluo-4 and Rhod-2 labeled cells. In wild type cells, an increasing trend of mitochondrial-to-cytosolic ratio is observed over time at both the IS and the whole cell (**Figure 6B & C**). In contrast, mitochondrial-to-cytosolic ratio shows no change after the IS formation in the *fat-1* cells, both at the IS and the whole cell (**Figure 6D & E**). Comparison between the mitochondrial-to-cytosolic ratio of wild type and *fat-1* cells at 15 min was not significant ($p>0.05$) at both the IS and the whole cell level. In contrast, at 30 min, significant differences ($p<0.05$) were observed in the mitochondrial-to-cytosolic ratio of wild type versus *fat-1* cells at the IS and the whole cell (**Figure 6E**). These data are consistent with an inhibitory effect of n-3 PUFA on the uptake of calcium by mitochondria after IS formation.

Figure 6. Effect of n-3 PUFA on intracellular Ca^{2+} . CD4^+ T cells were coincubated with Fluo-4 AM (3 μM) and Rhod-2 AM (2.5 μM) for 30 min and the mitochondrial-to-cytosolic Ca^{2+} ratio was determined. (A) Representative confocal microscopy images of Fluo-4 and Rhod-2 labeled cells (A1) white, (A2) RGB, (A3) green, (A4) red channels, respectively. (B) Amplitude of intracellular Ca^{2+} response at the immunological synapse (IS) in wild type CD4^+ T cells after co-culture with Hybridoma 145-2C11 antigen presenting cells. Data are from a representative experiment, 15-30 min after co-culture, n=5 independent experiments, 10-20 cells. (C) Amplitude of total intracellular Ca^{2+} response in wild type CD4^+ T cells. (D) Amplitude of intracellular Ca^{2+} response at the immunological synapse in *fat-1* CD4^+ T cells. (E) Amplitude of total intracellular Ca^{2+} response in *fat-1* CD4^+ T cells. (F) Comparison of relative mitochondrial-to-cytosolic Ca^{2+} ratio at the immunological synapse versus total cell in CD4^+ T cells from wild type (n=116 cells from 4 mice) and *fat-1* mice (n=131 cells from 5 mice). Asterisk indicates significant difference at $p<0.05$. (Left) Measurement at 15 min following the immunological synapse formation. (Right) Measurement at 30 min, the peak of response.

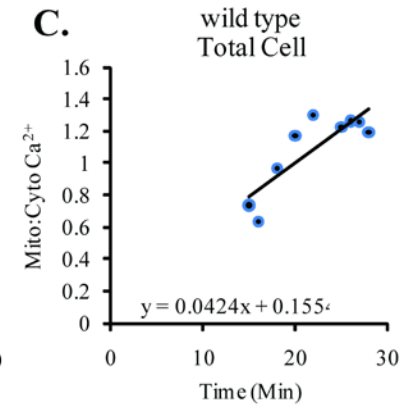
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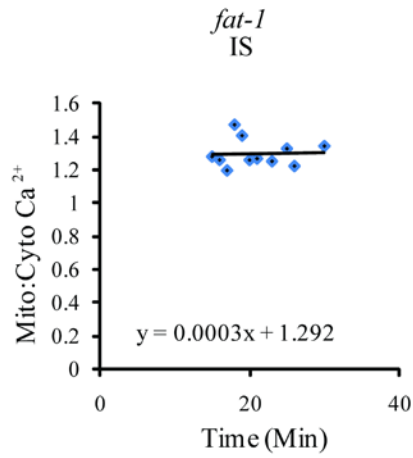
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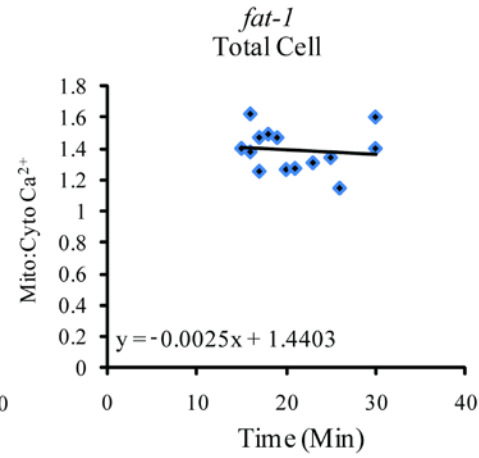
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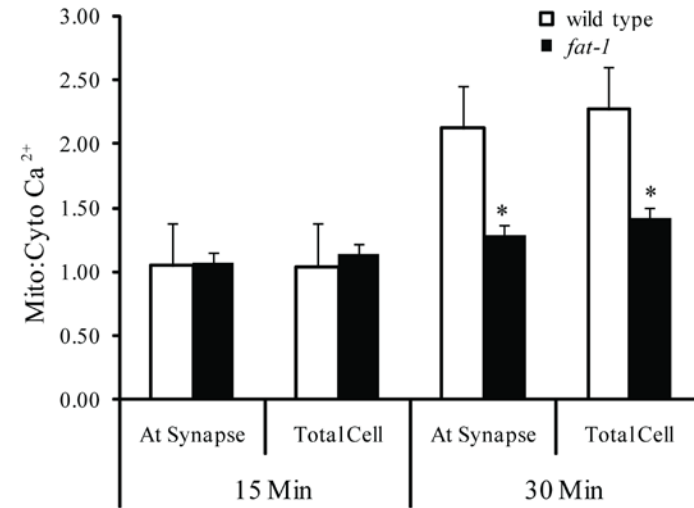
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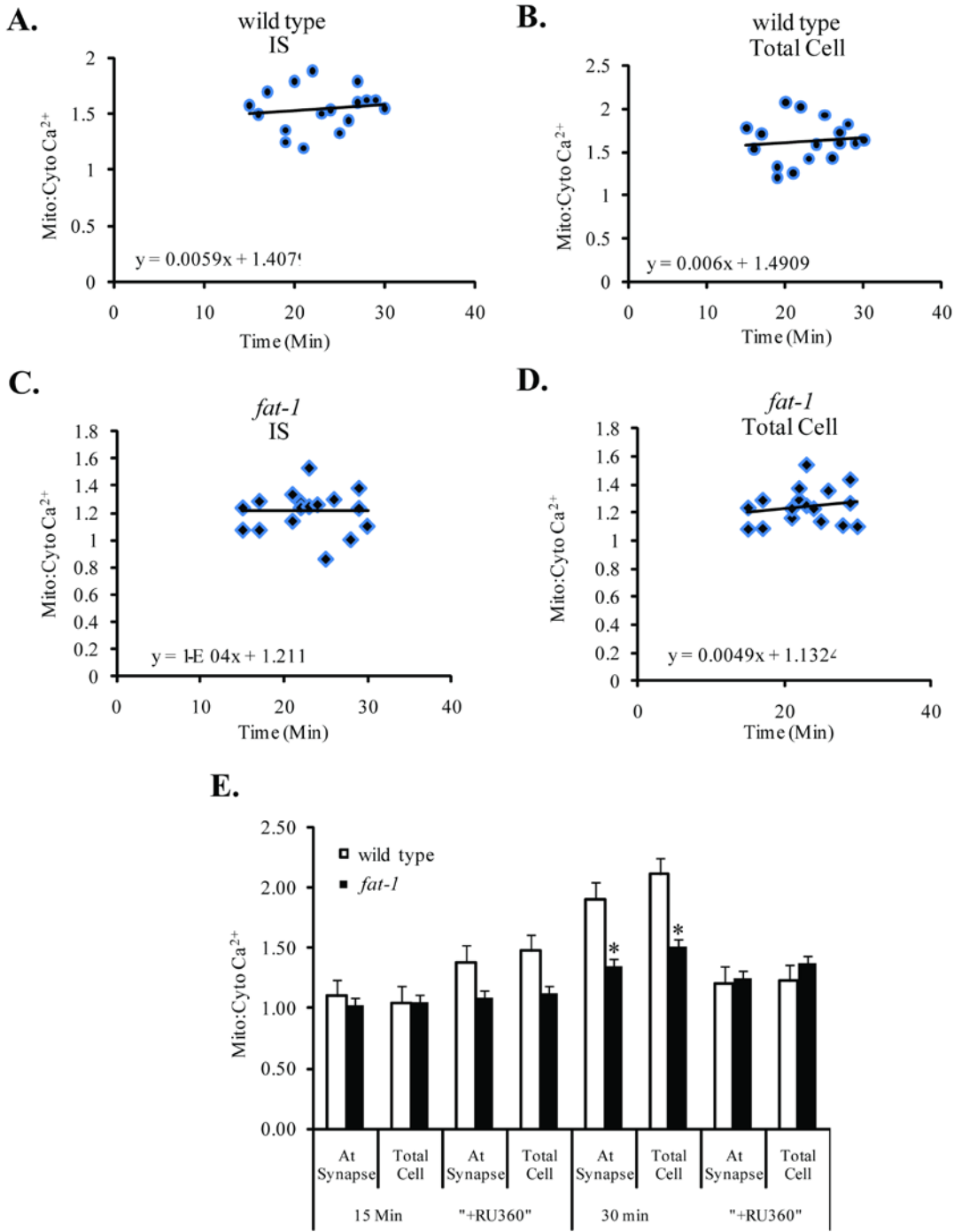
F.



3.4 Effect of a mitochondrial uniporter inhibitor on intracellular Ca^{2+} levels

To investigate the role of the mitochondrial uniporter in Ca^{2+} uptake, cells were treated with RU-360, a mitochondrial Ca^{2+} uniporter inhibitor (24). Following IS formation, the mitochondrial-to-cytosolic ratio in both the wild type and *fat-1* cells co-incubated with RU360 showed no change up to 30 min (**Figures 7A - D**). Significant differences ($p < 0.05$) in the mitochondrial-to-cytosolic Ca^{2+} ratio were observed in wild type cells and *fat-1* cells minus RU-360 both at IS and the whole cell at 30 min (**Figure 7E**). These data are consistent with the role of mitochondrial uniporter activity in regulating intracellular Ca^{2+} following IS formation.

Figure 7. Effect of mitochondrial uniporter inhibitor (RU-360) on intracellular Ca^{2+} . CD4^+ T cells from wild type and *fat-1* mice were preincubated with RU-360 (10 μM) for 30 min, followed by washing and coincubation with Fluo-4 AM (3 μM) and Rhod-2 AM (2.5 μM) for 30 min and the mitochondrial-to-cytosolic Ca^{2+} ratio was determined. (A) Amplitude of intracellular Ca^{2+} response at the immunological synapse (IS) with RU-360 in wild type CD4^+ T cells after 15 min co-culture with Hybridoma 145-2C11 antigen presenting cells. Data are from a representative experiment, 15-30 min after co-culture, n=2-3 independent experiments, 15-18 cells. (B) Amplitude of total intracellular Ca^{2+} response with RU-360 in wild type CD4^+ T cells after 15 min co-culture with Hybridoma 145-2C11 antigen presenting cells. (C) Amplitude of intracellular Ca^{2+} response at the immunological synapse with RU-360 in *fat-1* CD4^+ T cells. (D) Amplitude of total intracellular Ca^{2+} response with RU-360 in *fat-1* CD4^+ T cells. (E) Comparison of relative mitochondrial-to-cytosolic Ca^{2+} ratio at the immunological synapse vs. total cell in CD4^+ T cells from wild type (n=66 cells from 2 mice) and *fat-1* (n=92 cells from 3 mice). Asterisk indicates significant difference at $p < 0.05$. (Left) Measurement with and without RU-360 at 15 min following immunological synapse formation. (Right) Measurement with and without RU-360 at 30 min, the peak of response.



3.5 Effect of CRAC/ORAI1 channel blocker on intracellular Ca^{2+} levels

Since sustained Ca^{2+} influx across the plasma membrane through CRAC channels is required for T cell activation (25), the effects of BTP2, a CRAC/ORAI1 channel blocker, was evaluated in wild type CD4^{+} T cells. As shown in **Figures 8A – C**, the mitochondrial-to-cytosolic ratio at the IS and in the whole cell did not show any increase over time. These data suggest that inactivation of CRAC/ORAI1 channels inhibits the influx of extracellular Ca^{2+} preventing the increase in mitochondrial-to-cytosolic Ca^{2+} ratio necessary for downstream effector pathway activation.

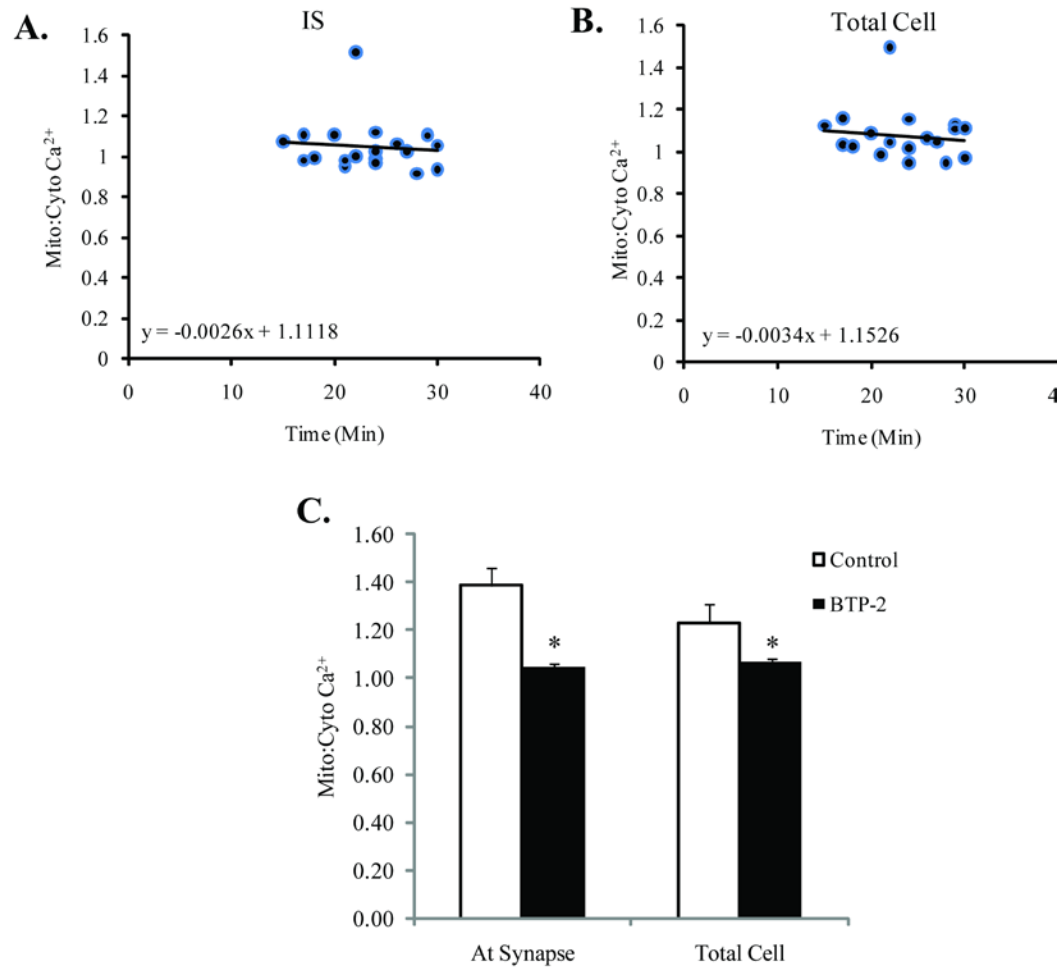


Figure 8. Effect of CRAC/ORAI1 channel blocker (BTP2) on intracellular Ca^{2+} . CD4^{+} T cells of wild type mice were preincubated with BTP2 (10 μM) for 1 h following which cells were washed and coincubated with Fluo-4 AM (3 μM) and Rhod-2 AM (2.5 μM) for 30 min and the mitochondrial-to-cytosolic Ca^{2+} ratio was determined. (A) Amplitude of intracellular Ca^{2+} response at the immunological synapse (IS) with BTP2 in wild type CD4^{+} T cells after a 15 min co-culture with Hybridoma 145-2C11 antigen presenting cells ($n=15-19$ cells from 15 to 30 min following IS formation). (B) Amplitude of total intracellular Ca^{2+} response with BTP2 in wild type CD4^{+} T cells. (C) Comparison of relative mitochondrial-to-cytosolic Ca^{2+} ratio at the immunological synapse versus total cell in wild type CD4^{+} T cells ($n=35$ cells). Asterisk indicates significant difference at $p < 0.05$.

CHAPTER IV

DISCUSSION AND CONCLUSION

Autoimmune and/or chronic inflammatory diseases primarily involve an overactive immune response directed towards a particular tissue (26). Improper T cell regulation results in an imbalance between T cell activation and suppression resulting in chronic inflammation. For example, hyperactivation of CD4⁺ T cells is associated with enhanced susceptibility to autoimmune disorders and chronic inflammation induced diseases (27). A plethora of studies demonstrate that T cell activation is suppressed by n-3 PUFA/DHA, in part, by altering the physical properties of biological membranes (10-13, 18, 28, 29). To further investigate the immunomodulatory effects of n-3 PUFA, in vivo mitochondrial translocation and calcium homeostasis in T lymphocytes following immunological synapse formation were investigated. There is evidence that mitochondria translocate toward the immunological synapse in CD4⁺ and Jurkat T cell lines (5, 25, 30). Recently, the re-organization of mitochondria at the NK cell synapse has been documented (31).

To our knowledge this is the first study to investigate the effects of n-3 PUFA on critical early Ca²⁺-mediated events of T cell activation. For this purpose, *fat-1* transgenic mouse model was used to test the hypothesis that n-3 PUFA inhibit mitochondrial translocation and, therefore, suppress sustained Ca²⁺ signaling necessary for T cell activation. The *fat-1* transgenic mouse model was utilized because it is capable of converting n-6 PUFA to n-3 PUFA endogenously by introducing a cis-double bond into

fatty acyl chains (14, 17). This model allows us to investigate the biological properties of n-3 PUFA without having to incorporate the fatty acid in the diet.

Recent evidence indicates that upon initial contact between APCs and T cells, mitochondria are recruited to the immediate vicinity of the immunological synapse (5). This critical event is followed by a reduction in the local accumulation of Ca^{2+} so as to maintain the robust influx of Ca^{2+} across the plasma membrane through CRAC channels, resulting in the activation of key downstream transcription factors such as NFAT (5). Previously it has been demonstrated that incorporation of n-3 PUFA into cytofacial leaflet phospholipids alters the lateral composition of lipid rafts in the plasma membrane, thereby altering the IS microenvironment to impact thresholds for the activation of TCR-mediated cell signaling. Thus, n-3 PUFA-enriched CD4^+ T cells exhibit a suppressed localization of important signaling proteins, such as F-actin, $\text{PLC}\beta\text{-1}$, and $\text{PKC}\theta$, into the IS as assessed by immunofluorescence labeling (13). Therefore, it was hypothesized that n-3 PUFA would suppress CD4^+ T cell mitochondrial translocation during the early stages of IS formation and down-modulate Ca^{2+} dependent signaling. Our data demonstrate that n-3 PUFA suppress the activation of T cells, in part, by inhibiting mitochondrial translocation to the immunological synapse. Specifically, experiments showed that CD4^+ T cells from *fat-1* mice had a significantly reduced percentage of cells that exhibited mitochondrial translocation to the IS as compared to cells isolated from wild type mice.

The sustained activity of Ca^{2+} channels in the plasma membrane requires translocation of mitochondria to the plasma membrane (5). This is attributed to the fact

that the decreased distance between mitochondria and the plasma membrane enables mitochondria to take up large amounts of extracellular Ca^{2+} , thereby preventing Ca^{2+} -dependent inactivation of CRAC channels, and sustaining Ca^{2+} -dependent signaling. Since mitochondrial translocation was inhibited in T cells isolated from *fat-1* transgenic mice, further study was done to examine the effect of n-3 PUFA enrichment on intracellular Ca^{2+} homeostasis in T cells using a double excitation technique, i.e., excitation at two wavelengths (488 nm and 550 nm) to determine increases in $[\text{Ca}^{2+}]_i$. This technique corrects for errors that are caused by the shift in the spectrum from one wavelength to another during increases in intracellular Ca^{2+} (20). As anticipated, the inhibition of mitochondrial translocation in *fat-1* T cells was associated with a reduction in mitochondrial Ca^{2+} uptake near the IS. It was also noted that the mitochondrial-to-cytosolic Ca^{2+} ratio remained unchanged in n-3 PUFA enriched (*fat-1*) T cells following IS formation. In contrast, wild type cells showed an increasing trend with respect to the mitochondrial-to-cytosolic ratio Ca^{2+} over time at both the IS and the whole cell. These data are noteworthy, because Ca^{2+} mediated regulation of CRAC channels in T cells enable sustained elevation of intracellular Ca^{2+} , an obligate requirement to drive nuclear translocation of NFAT (32). Maintenance of nuclear localization of NFAT is a prerequisite for the expression of interleukin-2 and other T cell activation responses after exposure to antigen (33, 34). In the absence of mitochondrial translocation and, hence, mitochondrial Ca^{2+} uptake, the local accumulation of Ca^{2+} near the IS inactivates CRAC/ORAI1 channel quickly and reduces Ca^{2+} entry into the cell (5). Our results reaffirm the importance of mitochondrial translocation, and demonstrate for the first

time that n-3 PUFA are capable of decreasing mitochondrial Ca^{2+} uptake, thereby impairing a universal second messenger pathway important for T cell activation.

To address the integration and crosstalk between Ca^{2+} and other signaling pathways in our T cell model system, proof-of-principle experiments were also conducted using mitochondrial uniporter (RU-360) and CRAC/ORAI1 channel (BTP2) inhibitors. As anticipated, following immunological synapse formation, mitochondrial-to-cytosolic Ca^{2+} ratios in both wild type and *fat-1* cells co-incubated with RU-360 were similar. In contrast, in the absence of RU360, the ratio was elevated only in wild type cells. In complementary experiments, inactivation of the CRAC/ORAI1 channel prevented the synapse-induced increase in the mitochondrial-to-cytosolic Ca^{2+} ratio. These data corroborate the functional link between mitochondrial regulation of CRAC/ORAI1 and T cell activation. Our results stress the importance of mitochondria in regulating Ca^{2+} channel activity and signal transmission from the plasma membrane to the nucleus during the earliest stages of T cell activation. Therefore, the immunosuppressive effects of n-3 PUFA on T cell function can be attributed, in part, to modulation of mitochondria-dependent intracellular Ca^{2+} signaling.

Although previous studies have examined the effects of diet with regard to immunosuppression (27, 35, 36), it is apparent that n-3 PUFA profoundly reduce the translocation of mitochondria to the immediate vicinity of the IS (**Figure 9**). This, in turn, reduces Ca^{2+} uptake by mitochondria, diminishing the “local” cytosolic Ca^{2+} concentration, which limits Ca^{2+} influx through CRAC/ORAI1 channels. Ultimately, lower intracellular Ca^{2+} inactivates the phosphatase calcineurin, and reduces the

subsequent nuclear import and assembly of NFAT transcription complexes essential for T cell activation (3, 4, 34). With regard to mechanisms which modulate mitochondrial motility, there is evidence that n-3 PUFA can displace F-actin from the IS, where T cells and APC form a conjunction (13, 28). This is noteworthy, because mitochondria predominantly interact with the actin cytoskeleton and use actin tracks to coordinate the relative distance between the IS and mitochondria (5, 37, 38). Further work is needed in order to dissect the effects of membrane lipid composition on microtubule based mitochondrial mobility and the overall actin morphology that provides the dynamic cellular framework that is required to facilitate several complex cytoskeleton-dependent processes and ultimately control T cell activation.

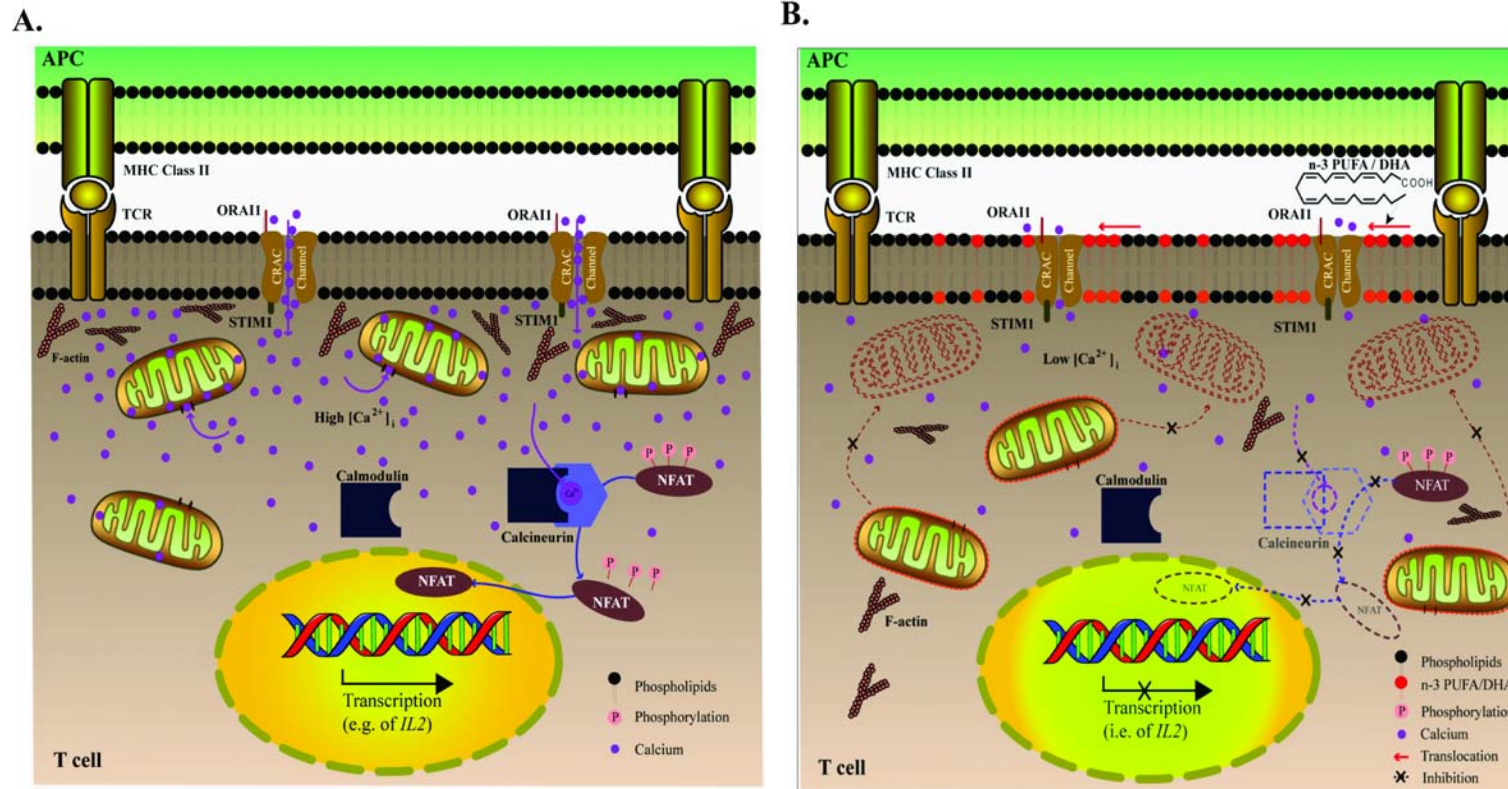


Figure 9. Effect of n-3 PUFA on Ca^{2+} -dependent T cell activation. (A) Schematic model showing the role of mitochondria during T cell activation. Upon formation of the IS between an APC and T helper (Th) cell, mitochondria translocate to the immediate vicinity of the IS. Once recruited to the IS, mitochondria reduce local Ca^{2+} accumulation, thereby sustaining Ca^{2+} influx through CRAC/ORAI1 channels. Increase in the intracellular calcium $[Ca^{2+}]_i$ concentration leads to calcium and calmodulin binding to the calcium-calmodulin-dependent phosphatase, calcineurin. Calcineurin then dephosphorylates transcription factors, e.g., NFAT, which translocate to the nucleus and remain in a transcriptionally active state. (B) The incorporation of n-3 PUFA into the *fat-1* $CD4^+$ T cell plasma membrane increases the formation of lipid rafts at the IS and also alters the composition of mitochondrial membrane. This results in the inhibition of mitochondrial translocation to the IS, thereby reducing Ca^{2+} uptake by mitochondria, limiting the cytosolic Ca^{2+} concentration, and blocking phosphatase activation of calcineurin. Collectively, these events reduce the dephosphorylation and nuclear translocation of NFAT and suppress the transcription of genes necessary for T cell activation.

In conclusion, these data demonstrate that n-3 PUFA suppress mitochondrial translocation to the immunological synapse and perturb Ca^{2+} signaling, which is critical to T cell activation. These results provide a critical new paradigm in understanding the molecular mechanisms through which n-3 PUFA modulate T-cell activation. The series of events that control Ca^{2+} signaling in T cells is complex and more research will be needed to understand and to elucidate the precise molecular mechanism by which dietary n-3 PUFA modulate $[\text{Ca}^{2+}]_i$ in T cells, and control several key activation events that lead to the production of pro-inflammatory cytokines (TNF α , IL-1 α , and IL-6) and eicosanoids. Additional study need to be conducted to reveal that n-3 PUFA decreases NK cell activity, cytotoxic T lymphocyte activity, expression of the high affinity IL-2 receptor α gene in activated lymphocytes, lymphocyte proliferation, and the production of IL-2. Furthermore the work was restricted to a transgenic animal model and there is an obvious need to examine these effects and mechanisms in the context of diet, where dose of active fatty acids available is of critical importance.

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APPENDIX

EXPERIMENTAL PROTOCOLS

A-1. *Fat-1* phenotyping

*Always keep the samples in ice.

I. D-1: Prepare leak proof tubes

1. Put 1ml acetone in 12ml glass tubes with black caps (Teflon lined).
2. Evaporate for 1hrs in 80 deg.C. oven.
3. Check if acetone has evaporated.

II. D-0: Extract total lipid

1. Preset centrifuger at 4 deg.C.
2. Clean 12ml tubes
 - Do in hood
 - Pour out acetone (into brown bottle)
 - Put upside down on the rack (don't mix the caps)
 - Clean the tubes with 1ml methanol (GC grade). Use glass pipette
3. Put 1ml cold 0.1M KCL with plastic pipette into 2ml epi-tubes.
4. Cut the mouse tail to small pieces, into the tube from #3.
5. Homogenize the sample with Polytron blade (Ultra-Turrax T8, IKA Labortechnik) at speed 5 for 15 sec. (Put the sample tube on ice during the homogenization). The tail mixture will turn brownish color.
6. Add 5ml of Folch ($\text{CHCl}_3/\text{MeOH}$, 2:1, v/v) into 12ml glass tube, then transfer

the sample to the tube.

7. Vortex the sample for 1min.
8. Centrifuge the sample at 3000rpm for 5min at 4deg.C. (Put the glass tube inside plastic tube wrapper to avoid breaking during the centrifugation). After centrifugation, set centrifuger temp. at 20 deg.C.
9. Transfer lower phase to a leak-proof 12ml glass vial with Pasteur pipette. Put the bulb to make bubbling during passing the upper phase to avoid contamination. After getting out the pipette, throw away 1-2 drops.
10. Wipe the needles of N₂ dryer with MeOH in the hood.
11. Dry sample under N₂
12. Mix 6% HCl-MeOH under the hood near walk in freezer. (HCl is under the hood, and GC-grade MeOH is near the balance). Use mass cylinder to measure.
13. Add 3ml 6% HCl-MeOH to the tube.
14. Flush sample with N₂, close cap tight.
15. Vortex and incubate at 76 dec.C. Oven for ~15 hrs.
16. Prepare 4ml tubes with labels. (Recap with green Teflon caps).

III. D+1: Extract FAME

1. At this time, turn on GC
2. Set centrifuger at 4 deg.C.
3. Extract FAME by adding 1ml, cold 0.1M KCl and 2ml Hexane to the methylated sample. Vortex for 1min, centrifuge at 3000rpm for 5min at

4deg.C.

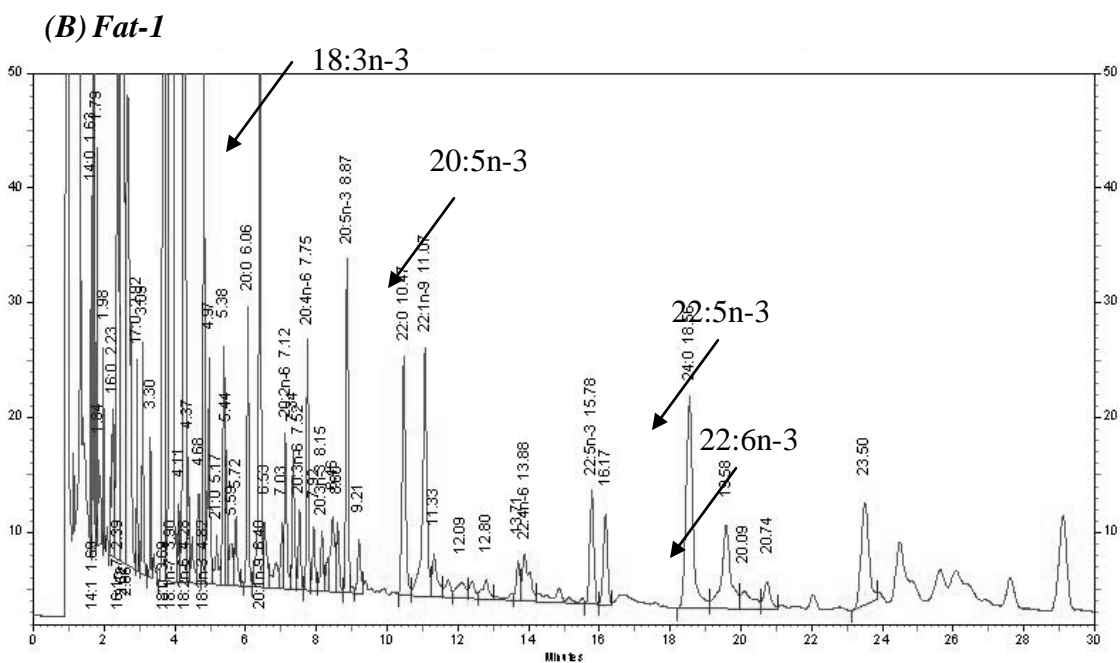
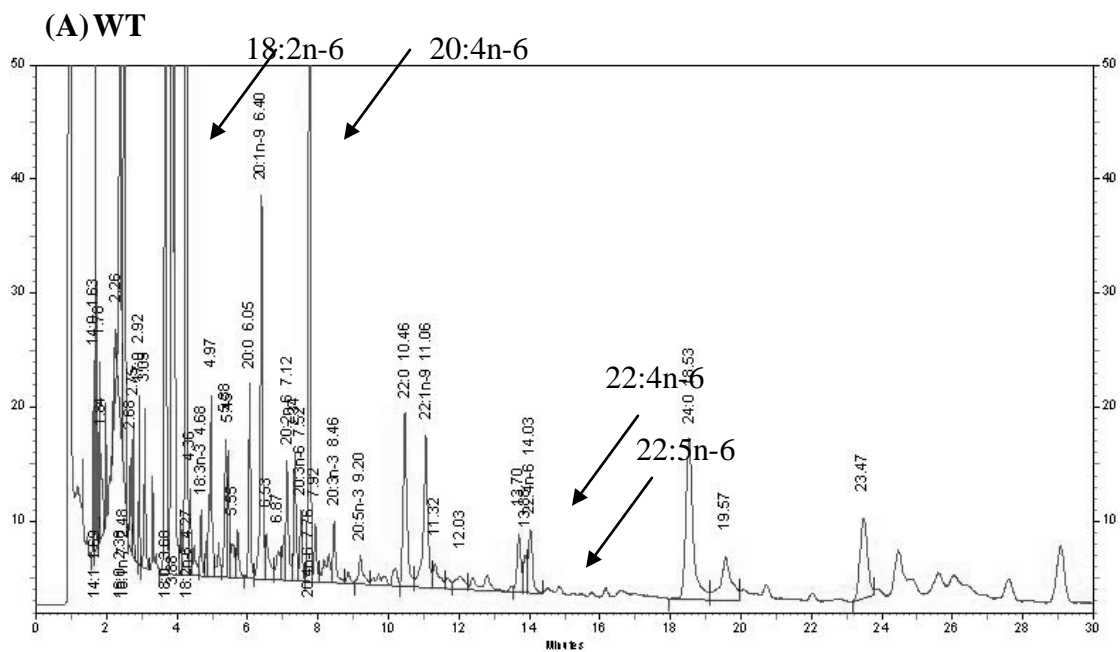
4. Transfer upper phase to 4ml glass vial.
5. Dry FAME under N₂.
6. Redissolve the sample in 25ul methylene chloride (CH₂Cl₂)
 - Pour CH₂Cl₂ into glass tube.
 - Use Wire Trol 25ul.
7. Vortex the tubes in order to dissolve all the FAME on the wall.
8. Ready for running on GC.

IV. GC analysis

1. Turn on GC, stabilize for 15min.
2. Turn on computer, open 32 Karat 7.0
3. Select GC, the OK.
4. Click on single run icon (Blue single arrow), the screen shows “waiting for trigger”
5. Then, inject std (68A std, Nuchek)
 - Find the tube with yellow tag in -20deg.C. freezer
 - Vortex
 - Rinse the inject needle with CH₂Cl₂
 - Load 0.5ul std in the needle: avoid any bubble.
 - Push the needle into injector inlet, the push the plunger.
 - Pull back the needle, then press “start” button on GC machine.
 - After injection, flush the tube with N₂.

- Put back in -20deg.C. freezer.
6. Vortex the sample and inject in the same way.
7. For the report,
- Click “3.2 Karat”, then “offline GC”.
 - Data → Open (D\GC\GCdata\wooki).
 - Method → integration events → minimum area → adjust → green (analyze).
 - Ctrl+z: escape highlight.
 - Method → custom report.
 - Double click on graph to change the Y axis range.

A-2. Representative GC profiles of *Fat-1* phenotyping



ID	Sex	n-6					n-3					n-6:n-3 ratio	Phenotype
		18:2	20:4	22:4	22:5	Total n-6	18:3	20:5	22:5	22:6	Total n-3		
974	F	19.7	1.2	0.4	0.3	21.6	2.1	1.7	1.2	1.3	6.3	3.4	<i>Fat-1</i>
975	F	19.0	1.2	0.6	0.4	21.2	1.7	1.6	1.5	1.1	5.9	3.6	<i>Fat-1</i>
979	F	19.2	1.7	0.7	0.6	22.2	1.5	1.5	1.4	1.3	5.7	3.9	<i>Fat-1</i>
980	F	11.8	1.2	0	0	13.0	2.7	1.8	1.6	1.6	7.7	1.7	<i>Fat-1</i>
962	M	15.8	6.6	1.5	1.3	25.2	0.0	0.0	0.0	0.0	0.0		WT
968	M	13.7	6.1	1.6	1.2	22.6	0.0	0.0	0.0	0.0	0.0		WT
1132	F	17.3	1.3	1.0	0.6	20.2	1.7	1.8	1.3	1.0	5.8	3.5	<i>Fat-1</i>
1133	F	16.2	1.2	0.6	0.0	18.0	2.5	1.3	1.2	1.7	6.7	2.7	<i>Fat-1</i>
1179	F	19.9	1.1	0.5	0.4	21.9	1.6	1.5	1.1	1.1	5.3	4.1	<i>Fat-1</i>
1180	F	13.8	2.6	0	0	16.4	2.2	1.8	1.8	1.4	7.2	2.3	<i>Fat-1</i>
742	M	19.6	1.2	0.6	0.4	21.8	1.3	1.4	1.1	1.0	4.8	4.5	<i>Fat-1</i>
991	M	15.8	1.5	0.4	0.0	17.7	1.8	1.2	1.1	1.3	5.4	3.3	<i>Fat-1</i>
998	M	16.3	1.3	0.5	0.0	18.1	2.1	1.1	1.0	1.2	5.4	3.4	<i>Fat-1</i>
999	M	9.7	0.6	0.3	0.2	10.8	1.1	0.9	0.9	0.6	3.5	3.1	<i>Fat-1</i>
961	M	18.9	1.5	0.5	0.4	21.3	1.4	1.4	1.5	1.3	5.6	3.8	<i>Fat-1</i>
1243	M	17.8	1.7	0.5	0.4	20.4	1.7	3.5	1.6	1.2	8.0	2.6	<i>Fat-1</i>
824	M	12.6	1.3	0.6	0	14.5	2.7	2.3	1.6	1.4	8.0	1.8	<i>Fat-1</i>
973	M	11.3	0	0.7	0.2	12.2	3.3	2.6	1.8	1.5	9.2	1.3	<i>Fat-1</i>
967	F	19.1	5.4	1.5	1.2	27.2	0.2	0.0	0.0	0.0	0.2	136.0	WT
971	F	18.7	0.6	1.5	1.3	22.1	0.5	0.0	0.0	0.3	0.8	27.6	WT
963	F	22.1	6.3	1.3	1.0	30.7	0.3	0.0	0.0	0.3	0.6	51.2	WT
1175	F	20.2	5.6	1.4	1.3	28.5	0.2	0.0	0.0	0.0	0.2	142.5	WT
1267	F	18.3	6.8	1.6	1.7	28.4	0	0	0	0	0.0		WT
982	M	16.2	5.9	1.5	1.2	24.8	0.3	0.0	0.0	0.3	0.6	41.3	WT
983	M	18.7	5.7	1.5	1.3	27.2	0.0	0.0	0.0	0.0	0.0		WT
1268	F	25.4	1.4	0.7	0.5	28.0	0.8	1.0	0.8	0.6	3.2	8.8	<i>Fat-1</i>
1269	F	16.2	1.1	0.5	0.0	17.8	1.9	1.4	1.2	1.6	6.1	2.9	<i>Fat-1</i>
1270	F	20.1	0.7	0.5	0.0	21.3	2.1	0.9	0.8	1.1	4.9	4.3	<i>Fat-1</i>
1244	M	8.5	0.6	0.9	0.0	10.0	3.2	2.0	1.5	2.0	8.7	1.1	<i>Fat-1</i>
1266	M	12.8	1.8	0.7	0.4	15.7	1.8	1.4	1.2	1.3	5.7	2.8	<i>Fat-1</i>

A-3. *Fat-1* genotyping

I. Isolation of total DNA from mouse tail

1. Material

- a. Mouse tail (~1cm), frozen in liquid nitrogen
- b. DNeasy Tissue Kit (Qiagen, cat# 69504)

2. Preparation of reagent

- a. For the first time use, add appropriate amounts of 100% EtOH to Buffers AW1 and AW2 as indicated on the bottles.
- b. Prewarm 55 °C. shaking water bath (set as 75 rpm)

3. Procedure (It is better to do this in the late afternoon)

- a. Perform all steps in RT, vortex for 5-10 sec. unless specified
- b. Allow the frozen tail warm to RT. Put the tail on a new glass slide.
- c. Cut the tail to smaller pieces using brand new or DNA away-zapped razor blade.
- d. Transfer the tail pieces to a new 2mL sterile epi-tube.
- e. Add 180uL Buffer ATL into the tube.
- f. Add 20uL proteinase K into the tube and vortex (make sure all pieces are down)
- g. Incubate the tube (on a floating platform) in 55 °C. shaking water bath (75 rpm) overnight (~16-18 hr).

----- Overnight -----

- h. Mix 200uL Buffer AL and 200uL 100% EtOH to make Buffer ALE for each sample, using DNase free pipette-tips. (Make fresh)
- i. Remove the tube out of the water bath. Vortex for 15 sec.
- j. Add 400uL Buffer ALE to the sample, and mix vigorously by vortexing (make sure it mix thoroughly)
- k. Pipette the mixture to DNeasy Mini spin column placed in a 2mL collection tube (kit provided).
- l. Centrifuge at $>6000 \times g$ (8000 rpm) for 1 min. (Eppendorf centrifuge 5414D)
- m. Discard flow-through and collection tube.
- n. Place the DNeasy Mini spin column in a new 2mL collection tube (provided).
- o. Add 500uL Buffer AW1, and centrifuge for 1 min at $>6000 \times g$ (8000 rpm).
- p. Discard flow-through and collection tube.
- q. Place the DNeasy Mini spin column in a new 2mL collection tube (provided).
- r. Add 500uL Buffer AW2, and centrifuge for 3 min at $20,000 \times g$ (14,000rpm) to dry the DNeasy membrane. (Centrifuge 5417R) It is important to completely dry the membrane, since EtOH will interfere with subsequent reactions.
- s. Discard flow-through and collection tube.
- t. Place the DNeasy Mini spin column in a new sterile 2mL collection tube with lid (not provided). Pipette 200uL of Buffer AE directly onto the DNeasy membrane.

- u. Incubate in RT for 1 min, then centrifuge for 1 min at $>6000 \times g$ (8000 rpm) to elute the DNA
- v. Pipette 200uL of Buffer AE directly onto the DNeasy membrane, in the same 2mL tube.
- w. Incubate in RT for 1 min, then centrifuge for 1 min at $>6000 \times g$ (8000 rpm) to elute the DNA in the same tube.

II. DNA quantification

1. Handle Quartz plate with lens cleaner, ALWAYS!! → Avoid “any” scratch !!
2. Turn on Spectra Max and the Mac computer connected. (Prewarm 10-15 min)
3. Find the “Spectra Max” icon on the Desktop, and double click it.
4. Click “Set up” → Wavelength Lm1: 260 / Lm2: 280 → Calibrate: On
5. Place Quartz plate on the machine
6. Find “Control” toolbar on menu → close tray
7. Control → Run → Pre-read → OK
8. The machine reads the plate, then the Quartz plate comes out.
9. Save, and bring the plate to the bench table.
10. Make 1X TE with 0.1% Tween 20
11. Put 95uL TE buffer into designated wells (duplicate is better)
12. Make it sure the first two wells are “Blank”
13. Use DNA quantification control ($\sim 115 \mu\text{g/mL}$, in Laurie’s freezer)
14. Put 5uL of DNA samples into appropriate wells.

15. Bring the plate to the Spectra Max, and load the plate.
16. Click Control → Close Tray → Read → Experiment → OK
17. The machine reads the plate
18. After the reading, click “Template”
19. Drag wells which are used → Unknown
20. Group number: 2
21. Drag first two wells → Blank
22. Then, look into result part
23. Delete any unnecessary columns
24. Calculate “MeanValues” for duplicates
25. Calculate “DNA quantity ($\mu\text{g/mL}$) by formulation: $\text{MeanValues} \times 20 (\text{dilution factor}) \times 214 (\text{Correction})$

Correction table

Volumn in Quartz plate	DNA	RNA
200uL	93.6	83.0
150uL	131.0	115.8
100uL	214.0	191.3
50uL	597.0	523.8

26. Dilute DNA to make 10uL of 15 $\mu\text{g/mL}$ DNA with ddH₂O. For PCR use only, discard diluent after use.

III. RT-PCR (SYBR Green real time PCR, 7900HT)

1. Per Reaction:

2X SYBR Master Mix	6.25 uL
Forward primer (300nM)	1uL
Reverse primer (300nM)	1uL
DNA (~15 µg/mL)	0.5uL
ddH ₂ O	3.75uL

2. Make a cocktail with Maser Mix, both primers, and ddH₂O.

Master Mix	6.25uL x	=	uL
F primer	1uL x	=	uL
R primer	1uL x	=	uL
ddH ₂ O	3.75uL x	=	uL
Total cocktail volume			uL

- a. Add 12uL of cocktail to plate and 0.5uL diluted DNA (Do in the hood for PCR, plate part# 4346906)
- b. Cover the plate with plastic cover.
- c. Squeeze with a rubber to ensure all wells are completely sealed. (If any well is not sealed, the material evaporates during PCR cycle).
- d. Centrifuge the plate several seconds. (When the centrifuge reaches up to 1000 rpm, turn off).
- e. Run RT-PCR

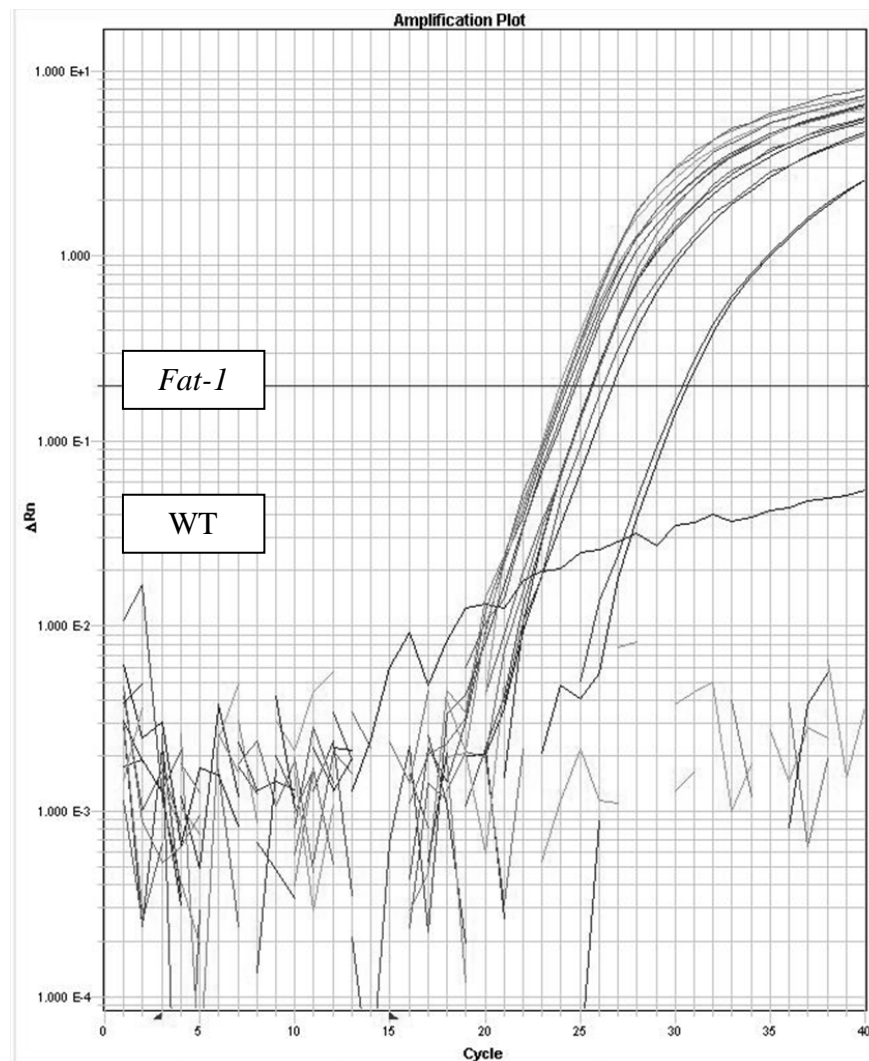
3. RT-PCR running

- a. Turn on machine and computer
- b. Click SDS 2.2.2 on desktop
- c. File → new
Assay: Absolute quantification
Container: 96 well
Template: Blank → OK
- d. Designate sample-containing wells by click and dragging on plate map
- e. (Use CTRL key if you need to add/drop wells)
- f. Setup → add detector → SYBR → copy to plate documents → done
- g. Mark “X” on detector SYBR
- h. (Make sure that all sample-containing wells are designated and marked as SYBR)
- i. Instrument → Mode → Standard → Sample volume(μ L) → 13.0
- j. Connect → open → load plate → close → start

4. After RT-PCR is done

- a. Designate wells → analysis → analyze
- b. (10) Open → take out sample → done

A-4. Representative RT-PCR plots of *Fat-1* genotyping



Primers used:

Fat-1 forward- tgt tca tgc ctt ctt ctt ttt cc

Fat-1 reverse- gcg acc ata cct caa act tgg a

Run PCR with following conditions:

- | | | |
|----------------------|---|------------|
| 1. 50 deg C : 2 min | } | 40 cycles. |
| 2. 95 deg C : 10 min | | |
| 3. 95 deg C : 15 sec | | |
| 4. 60 deg C : 1 min | | |

A-5. CD4⁺ T cell Positive Isolation by Miltenyi® Beads and Column

Materials:

- 70 μ m Cell Strainer (BD#352350, Fisher# 08-771-2)
- 30 μ m MACS Pre-separation Filter (Miltenyi Biotec, cat# 130-041-407)
- CD4 (L3T4) Microbeads, mouse (Miltenyi Biotec, cat# 130-049-201)
- MACS Separation Columns, **MS** columns (Miltenyi Biotec, cat# 130-042-201)
- Octo MACS Separation Unit (Miltenyi Biotec, #008716)
- Auto MACS Running Buffer (MACS Separation Buffer, Miltenyi Biotec, cat# 130-091-221).

Procedure:

Keep MACS buffer cold (on ice), but perform the rest of procedure at RT except when indicated.

I. Preparation for the kill

1. Prepare media/tubes
 - a. Fill conical tubes with 3 mL MACS buffer.
 - b. Prepare scissors, forceps, 70% EtOH squeeze bottle, 5 mL syringes.

II. Kill/Co-culture

2. Isolate CD4⁺ T cells from spleens
 - a. Sacrifice mice by CO₂.
 - b. Place mice on their right side so that the left side faces you.
 - c. Apply alcohol to the abdomen area.

- d. Grab the skin of the abdomen with forceps and make a small incision.
- e. Peel back the skin/fur with fingers to expose the membrane underneath.
- f. Grab the membrane with forceps and cut the membrane to expose the organs.
- g. Remove the spleen (dark red organ) with forceps.
- h. Carefully remove as much fat from the exterior of the spleen as possible.
- i. Place a spleen in a 15 mL conical tube containing 3 mL MACS buffer, transfer to cell culture room.

III. Single cell suspension (Critical!!) Perform the following steps under sterile hood.**

- 1. Place the 70 μ m Cell Strainer inside a 100mm petri dish, add 5 mL MACS buffer to wet the membrane.
- 2. Transfer the spleen with the 3 mL MACS buffer onto the cell Strainer.
- 3. Use a plunger from 5 mL syringe to mesh (by gently push, not grinding) the tissue inside the petri dish (Keep the tissue pieces wet at all time), till only connective tissue left on the membrane.
- 4. Remove the cell strainer; transfer the 8 ml buffer (containing cells) into the original 15 mL conical tube. Rinse the cell strainer inside dish with ~ 5 mL of MACS buffer; combine with the original 8 mL buffer in the conical tube, to get all the cells.
- 5. Centrifuge the cell suspension at 300 x g for 10 min.
- 6. Aspirate the supernatant (by attached a plastic pipet tip on top of glass pipet to reduce the suction force).

7. Tap the bottom of tube to break the pellet and get a better suspension.
8. Add 8 mL MACS buffer to fully resuspend the cell pellet.
9. Place a 30 μ m MACS Pre-separation Filter on top of a 15 mL conical tube. Add 2 mL buffer to wet the filter. Transfer the 8 mL cell suspension onto the filter; add small volume at a time, slowly, to prevent clogging too fast. (Some connective tissues will remain on the filter membrane)
10. Remove the filter, close the lid. Gently invert the tube to get a good mixture. Transfer a small aliquot ($\sim 20 \mu$ L) of cell suspension to an epi-tube for counting*.
11. Centrifuge the remaining cell suspension at 300 x g for 10 min.
12. *While waiting for centrifugation, count cell number in a hemacytometer with (1) Trypan Blue (10 μ L cell + 80 μ L PBS + 10 μ L Trypan Blue) to assess percent dead cells and; with (2) Crystal Violet stain (10 μ L cells + 90 μ L Crystal Violet) to assess WBC #. (RBC will be lysed and nuclei of leukocytes will be stained blue (*may need to adjust the focus to see the blue stain*). <Some time, you may need to dilute the original cell suspension before adding Trypan Blue or Crystal Violet.> (*The cell number obtained from Trypan Blue and Crystal Violet normally is pretty close, you don't really need the crystal violet stain...*)
13. Or, just count the cell number by Coulter counter (set at 4 μ m cut off)

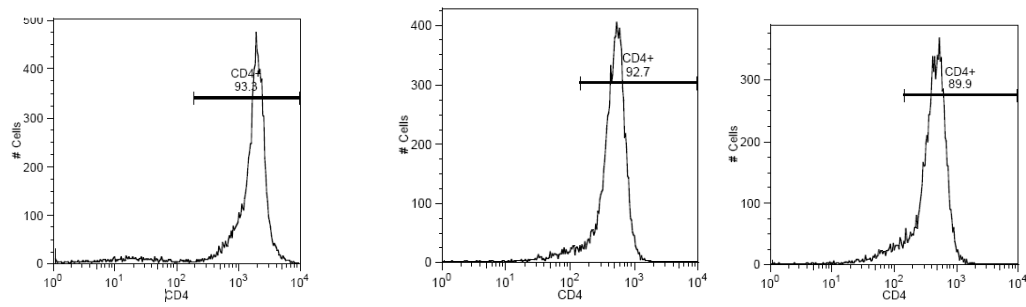
IV. CD4⁺ T cell isolation

1. Aspirate the supernatant (by attached a plastic pipet tip on top of glass pipet to reduce the suction force). Tap the bottom of tube to break the pellet and get a better suspension, then resuspend cells in appropriate amount of MACS buffer required in 15 mL conical tube (90 μ L cold MACS Buffer per 10^7 total cells** (should be primary WBC)) for labeling with MACS microbeads. (Load up to ~ **40 x 10^6 WBC/MS** column, too much will clog the column) *Record starting cell numbers for calculating yield later...*
2. Add 10 μ L of CD4 (L3T4) Microbeads per 10^7 total cells. Make sure to add the **microbead** directly **into the cells**; don't just add to the side of the tube.
3. Mix well by pipetting, and incubate for exactly 15 min at 4°C (refrigerator, not ice!!) (No shaking needed)
4. Wash cells by adding 1-2 mL of cold MACS buffer per 10^7 cells (*10 – 20 fold of cell suspension volume*), or, just fill the tube with MACS buffer; mix by inversion and centrifuge at 300 x g for 10 min.
5. Aspirate the supernatant (by attached a plastic pipet tip on top of glass pipet to reduce the suction force), tap the bottom of tube to break the pellet and get a better suspension. Resuspend in up to **500 μ L** cold MACS buffer per 10^8 cells.
6. Place MS column at Octo MACS Separator (the separator should be adhering to the stand on the vertical side). Orient the stick-outside of column against separator. Put a basin under the column for collecting waste.
- 7.

8. Prepare the following buffer volume is for **MS** column only.
 - a. Rinse MS column with 500 μ L cold MACS buffer. Let the buffer slowly drip to dry itself. (Very quick, less than 1 min, and it won't really dry completely)
 - b. Apply cell suspension onto the column.
 - c. Let the unlabeled cells pass through. Add 1.5 mL of cold MACS buffer to the sample tube (rinse to get all the cells), and wash MS column using the buffer inside the sample tube (3 times with 500 μ L each, let the reservoir empty between each wash). => The effluent is the unlabelled fraction, does not contain CD4⁺ T cells)
 - d. Remove MS column from the separator, and place it onto a collection 15 mL conical tube.
 - e. Pipette 1 mL of cold MACS buffer onto the column. Immediately flush out fraction with the magnetically labeled cells by firmly applying the plunger supplied with the column. Push the plunger into the column all the way, it will generate lots bubbles.
 - f. Centrifuge the elution (containing labeled cells) at 300 x g for 10 min. Resuspend the cell pellet in complete medium. => CD4⁺ population.
 - g. Count cell # with Trypan blue (for viability) or Coulter counter.

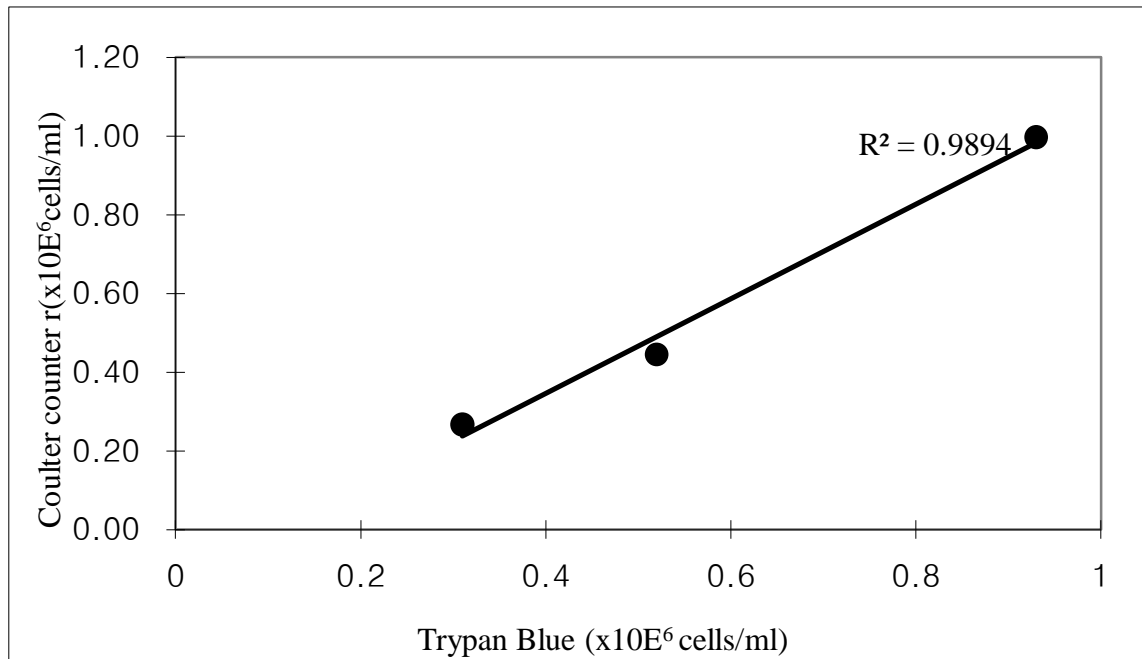
A-6. Purity of CD4⁺ T cells isolated using Miltenyi system

CD4⁺ T cell purity (C57BL/6, analyzed by BD FACSCalibur)



CD4⁺ T cell purity = 91.97 ± 1.05 % (n=3)

A-7. Comparison of cell count by hemocytometer and Coulter counter



Objective: To test if Coulter counter can be used for T cell quantification.

Methods: Purified CD4⁺ T cells by Miltenyi beads were resuspended in 1mL medium. Cells were subsequently counted by either Trypan Blue exclusion method or Coulter counter.

Results: The cell numbers counted by the two different methods correlate at $R^2=0.9894$.

I concluded that Coulter counter can be used to count CD4⁺ T cells.

A-8. Counting Cells with Coulter Counter

Prepare the vials with solution for counting the previous day and check the functioning of the counter

1. Turn power on, open door, lower stage.
2. Remove vial of Coulter Clenz, replace with Isotone/Isoflow, raise stage
3. Readout: Setup #1 (S1). Scroll down to last line and set cell size. This measurement is to be set for the smallest cell of acceptable range. For CD4⁺ cells, it is 4 μm .
4. Select: FUNCTIONS on keypad. Scroll to flush aperture. Hit: START/STOP to flush. Repeat flush, and then replace Isotone. Flush twice more.
5. Select: OUTPUT on keypad. Scroll to Count / Concentration and select one. If “concentration” is selected, readout will be in #cells/ml. Scroll to Dilution and enter dilution factor. Leave units on um.
6. Prepare sample cups 24-48 hours ahead: De-gas Isotone by turning red lever to the left. Lift dispensing pump twice. Turn red lever so it is parallel to the arm. Fill Coulter vials with 20 ml per vial (two pumps of Isotone).
7. Add 40 μl sample to Coulter vial (for 1:500 dil). Place vial on stage, raise, and press START/STOP on keypad to count. Count each sample 2-3 times and average.
8. To shut down machine, replace vial of blue Coulter Clenz and flush aperture (under FUNCTIONS) 2-3 times. When flush is complete, turn off power.

A-9. Counting Cells with Hemacytometer

A. Materials:

- i) Trypan blue (Sigma, cat# T8154).
- ii) Eppendorf tubes.
- iii) Hemacytometer.

B. Methods:

- i) Add 180 μL H_2O and 10 μL Trypan blue to an epi-tube.
- ii) Mix the T cells and remove 10 μL to add to a sample tube with Trypan blue.
- iii) Mix by pipetting up and down several times.
- iv) Remove about 20 μL from the suspension and put in a hemacytometer.
- v) Count the viable white cells by microscope at 10x magnification (count at 200 cells at least)

$$\text{Cell concentration (cells/mL)} = (\text{cell count}) / (\text{squares}) \times 10^4 \times 20$$

A-10. Hybridoma Handling (145-2C11, UC8-1B9)

I. Reagents

1. Complete medium

	Vol(mL)	%	Stock Conc	Final Conc	Vendor	Cat#
DMEM, high glucose formula	500.00	86.03			Gibco	11960-44
HI-FBS	58.00	9.98			Irvine Scientific	300320439
Glutamax	11.60	2.00	200mM	4mM	Gibco	35050-061
Na pyruvate (in cold room)	5.80	1.00	100mM	1mM	Cellgro	25-000-CI
Pen-Strep	5.80	1.00	10,000 U/mL P 10,000 ug/mL S	100 U/mL P 100 ug/mL S	Gibco	15140-148
Total	581.20	100.00				

2. Cryoprotectant medium: complete medium with 5% (v/v) sterile DMSO
3. hIL-6: During critical period (ie. After thawing from our lab depository in the N₂ tank), add 80u/ml hIL-6. hIL-6 stock is 200,000U/ml. Aliquots stored at -20 deg C. Eg: Mix 50ml complete media with 20ul hIL-6 (stock). (Roche, Cat# 11-138-600-001, Lot# 93320322).

4. Cell-line

145-2C11: ATCC Cat# CRL-1975 (Mouse anti-CD3 expressing hybridoma)

UC8-1B9: ATCC Cat# CRL-1968 (Mouse anti-DNP expressing hybridoma)

5. Handling frozen cells from ATCC

- a. For the 1st culture after purchase, Take 10 mL of complete media in 15 mL falcon tube.
- b. Fill one T-75 flask with complete medium (the volumes differ to each batch.
Confirm to Specific Batch Info)
- c. For culturing cells from our own frozen cells (2x10⁶ cells in 2 mL cryoprotective medium kept in liquid N₂ tank), prepare 18 mL complete media in T-75 flask.
- d. Place the flask into 5% CO₂ incubator at 37°C for at least 15min.
- e. Thaw the vial by gentle agitation in a 37 °C water bath. To reduce the possibility of contamination, keep the O-ring and cap out of the water.
Thawing should be rapid (approx. 2 min).
- f. Move the vial to sterile hood, and spray 70% EtOH to the vial.
- g. Transfer the thawed cell suspension into the 15 ml falcon tube prepared in step 1.
- h. Spin down (**100x g, 5 min**, critical) and aspirate supernatant to remove cryoprotective DMSO.
- i. Resuspend cells in 2 mL complete media.
- j. Transfer the cells to the pre-warmed T-75 flask prepared at step 3 or 4.
- k. Incubate in 5% CO₂ incubator at 37 °C.
- l. After thawing, cells become confluent in 3- 4 days.

- m. After 1st media change, cells become confluent and need to be passed every 2-3 d (If media look yellow-it means acidic-, pass cells immediately).
 - n. Count cell concentration by Coulter Counter. Adjust concentration to 1×10^6 cells/mL (for experiments) by adding complete DMEM media.
 - o. Generally, Raje used cells between 5rd to 12th passages for her immunofluorescence experiments.
6. Handling frozen cells from our lab N₂ Tank storage
- a. Prepare 18 mL complete media by adding 8 ul of hIL-6 in a T-75 flask and keep the flask in the 37°C incubator for ~15 min.
 - b. Take 10 mL of complete media in 15 mL falcon tube.
 - c. Thaw the vial by gentle agitation in a 37 °C water bath. To reduce the possibility of contamination, keep the O-ring and cap out of the water. Thawing should be rapid (approx. 2 min).
 - d. Move the vial to sterile hood, and spray 70% EtOH to the vial.
 - e. Transfer the thawed cell suspension into the 15 ml falcon tube prepared in step 2.
 - f. Spin down (**100x g, 5 min,** critical) and aspirate supernatant to remove cryoprotective DMSO.
 - g. Resuspend cells in 2 mL complete media.
 - h. Transfer the cells to the pre-warmed T-75 flask prepared at step 1.
 - i. Incubate in 5% CO₂ incubator at 37 °C.
 - j. After thawing, cells become confluent in 3- 4 days.

Note: Media change from now on will be in complete media; no hrIL-6; cells become confluent and need to be passed every 2-3 days (If media looks yellow-it means acidic-, pass cells immediately).

- k. Count cell concentration by Coulter Counter. Adjust conc. to 1×10^6 cells/mL (for experiments) by adding complete DMEM media.

7. Freezing cells to our lab N₂ Tank storage

- a. Prepare 18 mL complete media in a T-75 flask and keep the flask in the 37°C incubator for ~15 min.
- b. Harvest suspending cells by pipette into a 50 mL conical tube. (Try to avoid mixing adherent cells by shaking flask or touching the flask bottom).
- c. (Optional) If you need to keep cells in liquid N₂ tank for future study, take ~half volume of cell suspension into a separate 50 mL conical tube for freezing and the other half for future passages. Label the two falcon tubes. (Tube 1: Freezing, Tube 2: Passage).
- d. Take 10-20 µL for cell concentration by Coulter Counter (4 µm).
- e. Centrifuge cells at 300x g for 5 min. (50 mL falcon tube from step 3)
- f. Aspirate the supernatant and resuspend cells; adjusting the conc. to 1×10^6 cells/mL by adding complete DMEM with 5% sterile DMSO
- g. Aliquot 1 mL cell suspension containing 5% DMSO into 2.5 mL cryotubes, and label on tubes cell type, date, passage number, cell number and your initial. Freeze in a “Mr. Frosty” at -80°C overnight. Next day, move to liquid N₂ tank.

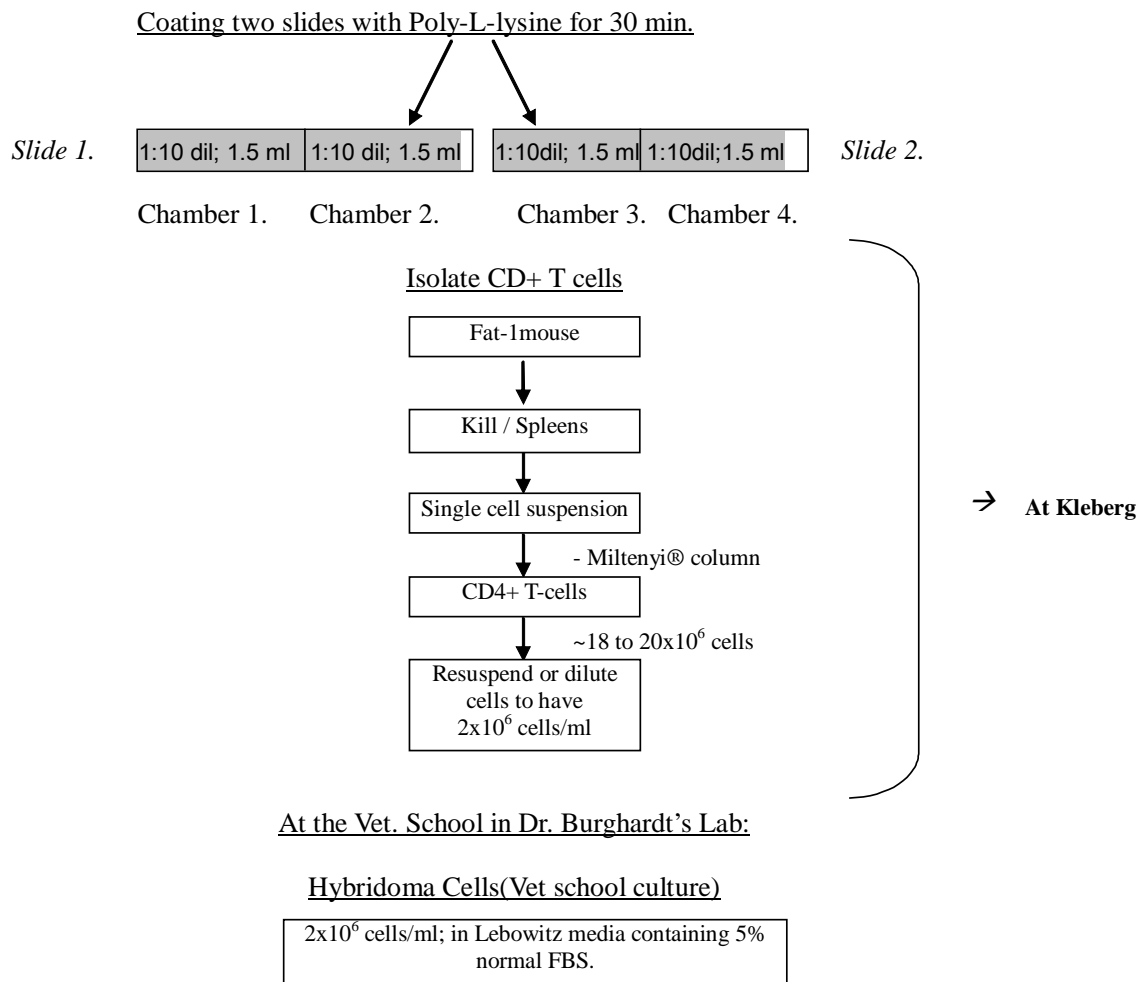
<For further passage >

- h. Transfer 2 mL cell suspension from the falcon tube 2; prepared and centrifuged in step 3-5; into the pre-warmed T-75 flask in step 1 of freezing cells.
- i. Place the flask in the 37°C 5% CO₂ incubator.

<Cell viability of adherent cells>

- j. (For 145-2C11 and UC8-1B9) A number of cells are adherent to the flask bottom. The viability of 145-2C11 was 93.66% (suspending cells) and 77.77% (adherent cells) determined by trypan blue exclusion method. Therefore, use only suspending cells by pipetting up the media, not touching the flask bottom.

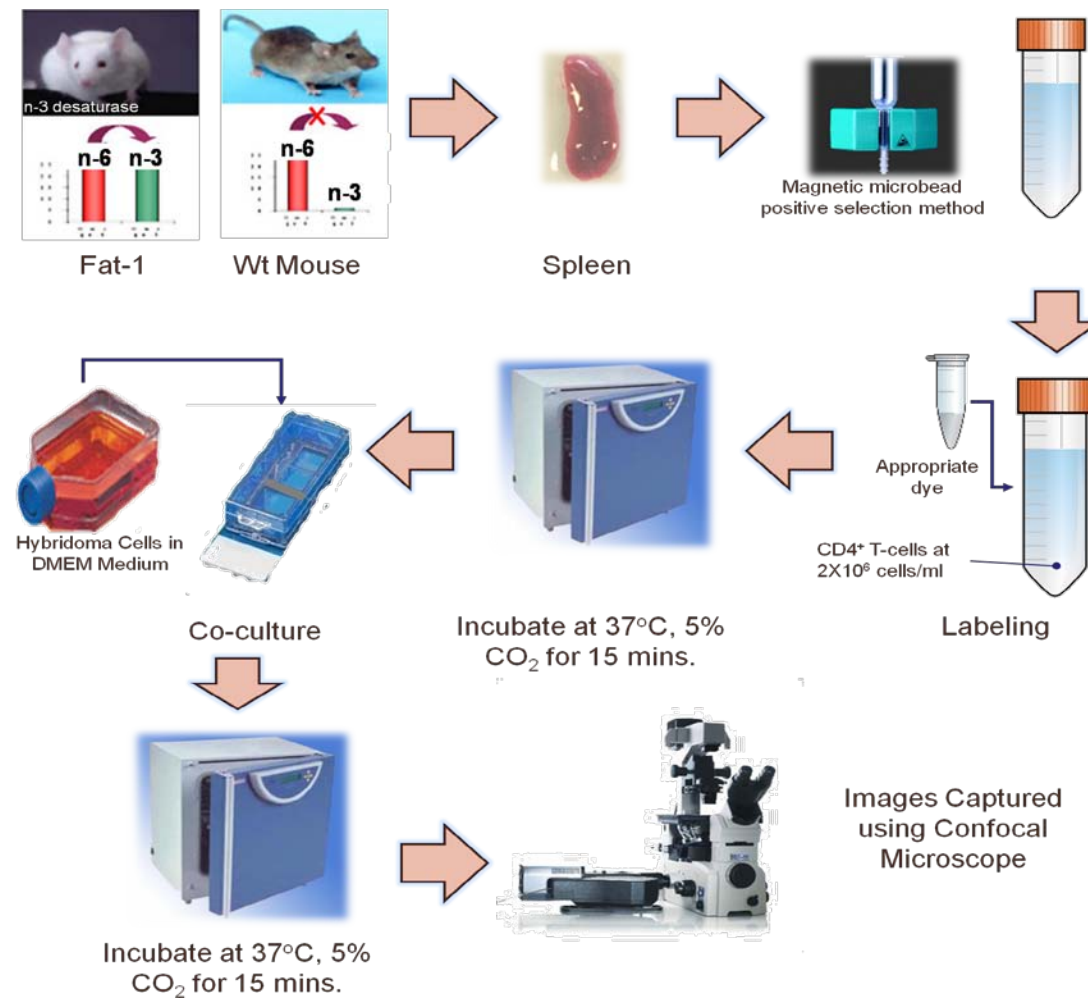
A-11. Experiment Plan



1. Prepare the calcium dye
2. Labeling T-cells with calcium dyes
3. Mix T-cell with Hybridoma(5:1)
4. Rinse the slides with 1ml Lebovitz's medium.
5. Transfer 1.2 ml cell suspension to chamber of different slides.
6. Image: Capture images after 15 min. incubation in 37 deg.C. 5%CO₂.

Report time of the image taken and copy images on pen drive and bring to Kleberg Lab 323 for analysis.

A-12. Diagrammatic representation of the Experimental Protocol



A-13. Mitochondria Imaging in T cells

- I. Preparation of poly-L-lysine (Sigma, cat#P8920)) coated chamber slides (Lab-tech chambered cover glass slides, 31 German Borosilicate, Nunc, and Cat #155380). 2 slides (4 chambers)
 1. Dilute 1ml poly-L-lysine in 9 ml sterile water.
 2. Dispense 1.5 ml of diluted poly-L-lysine into each well of 2 slides (4 chambers)
 3. Incubate for 30 minutes at RT.(leave in the hood and label: Do not move)
 4. Aspirate the solution and wash with 1 ml warm Leibovitz before using
- II. Isolate CD4+ T cells from spleens of WT/ fat-1 mice
 1. CD4+ T-cells at 2 million cells /ml/tube in RPMI media, incubate at 37°C with 5% CO₂ (*Loosen the caps of the tubes when in the incubator)
 2. Hybridoma (CD3+) at 2 million cells/ml – (*Cells in phenol free complete DMEM media)
 3. Mitochondrial dyes
 4. Leibovitz's medium
 5. 10p pipette/tips
 6. 200p pipette/tips
 7. 1000p pipette/tips
 8. Timer
 9. Poly-L-Lysine coated chamber slides

III. Dye Preparation

1. Prepare stock solutions of MitoTracker Green FM on the day of experiment

Stock preparation of 1 mM Mitotracker Green FM, Molecular Probes, M-7514

- a. Molecular weight of Mitotracker is 671.88 and the vial contains 50 µg.
- b. Dissolve 50 µg Mitotracker (vial) in 74.42 µl DMSO to make up 1 mM Mitotracker
- c. Mix well by pipetting up and down.
- d. 0.2 µl of Mitotracker green stock needs to be added to the 1ml of Leibovitz media for incubation to make a final concentration of 200 nM.

A-14. T cell labeling Mitochondrial localization

I. Labeling of Mitotracker for mitochondrial translocation

1. Add 0.2 uL of [1 mM stock] Mitotracker solution into 1ml CD4⁺ T cell suspension.
2. Vortex for 2 sec. Keep the lid of the tube loose.
3. Incubate at 37 deg.C. for 10 min.
4. Spin down at the speed 5 (Dr. Burghardt's centrifuge) for 5 min.
5. Suck out dye with the media.
6. Resuspend cells in 1 ml Leibovitz's medium.

II. Mix cells (T cell:hybridoma = 5:1)

1. Mix 0.2 mL hybridoma into 1ml T cell suspension.
2. Transfer 1.2 mL of cell suspension from the tube to 1 well of the chambered slide.
3. Visualize and see the best synapse. Capture images starting at "0" time after mixing with hybridoma cells and continue on for 30 min (1 image every 3 minutes for time kinetic).
4. Collect your images, report time after the experiment and bring to lab 323 in Kleberg. The images are analyzed using adobe photoshop on our 323 lab computer.

A-15. Measurement of Mitochondrial Ca^{2+} in T cells

I. Preparation of poly-L-lysine (Sigma, cat#P8920)) coated chamber slides (Lab-tech chambered cover glass slides, 31 German Borosilicate, Nunc, and Cat #155380). 2 slides (4 chambers)

1. Dilute 1ml poly-L-lysine in 9 ml sterile water.
2. Dispense 1.5 ml of diluted poly-L-lysine into each well of 2 slides (4 chambers)
3. Incubate for 30 minutes at RT.(leave in the hood and label: Do not move)
4. Aspirate the solution and wash with 1 ml warm Leibovitz before using

II. Isolate CD4^+ T cells from spleens of WT/ *fat-1* mice

1. CD4^+ T-cells at 2 million cells /ml/tube in RPMI media, incubate at 37°C with 5% CO_2 (*Loosen the caps of the tubes when in the incubator)
2. Hybridoma (CD3^+) at 2 million cells/ml – (*Cells in phenol free complete DMEM media)
3. Mitochondrial dyes
4. Leibovitz's medium
5. 10p pipette/tips
6. 200p pipette/tips
7. 1000p pipette/tips

8. Timer
9. Poly-L-Lysine coated chamber slides

III. Dye Preparation

1. Prepare stock solutions on the day of experiment

Stock preparation of 1 mM Mitotracker Green FM, Molecular Probes, M-7514

- a. Molecular weight of Mitotracker is 671.88 and the vial contains 50 μg .
- b. Dissolve 50 μg Mitotracker (vial) in 74.42 μl DMSO to make up 1 mM Mitotracker
- c. Mix well by pipetting up and down.
- d. 0.2 μl of Mitotracker green stock needs to be added to the 1ml of Leibovitz media for incubation to make a final concentration of 200 nM.

Stock preparation of 4 mM stock of Rhod-2, Molecular Probes, R1245

- a. Molecular weight of Rhod-2 is 1123.96 and the vial contains 50 μg .
- b. Dissolve 50 μg of Rhod-2 (vial) in 10 μl DMSO to make up 5 mM Rhod-2
- c. Mix well by pipetting up and down.
- d. 0.3 μl of Rhod-2 stock needs to be added to 1 ml Leibovitz media to make a final concentration of 1.5 μM

IV. T cell labeling Mitochondrial Ca^{2+} measurement

1. Labeling of Mitotracker, and Rhod-2 for mitochondrial Ca^{2+}
 - a. Add 0.5 uL of [5 mM stock] Rhod-2 into 1ml CD4^{+} T cell suspension.
 - b. Vortex for 2 sec.
 - c. Incubate at 37 deg.C. for 15 min.
 - d. Add 0.2 uL of [1 mM stock] Mitotracker solution into 1ml CD4^{+} T cell suspension.
 - e. Vortex for 2 sec. Keep the lid of the tube loose.
 - f. Incubate at 37 deg.C. for 15 min.
 - g. Spin down at the speed 5 (Dr. Burghardt's centrifuge) for 5 min.
 - h. Suck out dye with the media.
 - i. Resuspend cells in 1 ml Leibovitz's medium.
2. Mix cells (T cell:hybridoma = 5:1)
 - a. Mix 0.2 mL hybridoma into 1ml T cell suspension.
 - b. Transfer 1.2 mL of cell suspension from the tube to 1 well of the chambered slide.
 - c. Visualize and see the best synapse. Capture images.
 - d. Collect your images, report time after the experiment and bring to lab 323 in Kleberg. The images are analyzed using adobe photoshop on our 323 lab computer.

A-16. Measurement of mitochondrial-to-cytosolic Ca^{2+} ratio using Rhod-2 and

Fluo-4

I. Preparation of poly-L-lysine (Sigma, cat#P8920)) coated chamber slides (Lab-tech chambered cover glass slides, 31 German Borosilicate, Nunc, and Cat #155380). 2 slides (4 chambers)

1. Dilute 1ml poly-L-lysine in 9 ml sterile water.
2. Dispense 1.5 ml of diluted poly-L-lysine into each well of 2 slides (4 chambers)
3. Incubate for 30 minutes at RT.(leave in the hood and label: Do not move)
4. Aspirate the solution and wash with 1 ml warm Leibovitz before using

II. Isolate CD4^{+} T cells from spleens of WT/ *fat-1* mice

1. CD4^{+} T-cells at 2 million cells /ml/tube in RPMI media, incubate at 37°C with 5% CO_2 (*Loosen the caps of the tubes when in the incubator)
2. Hybridoma (CD3^{+}) at 2 million cells/ml – (*Cells in phenol free complete DMEM media)
3. Mitochondrial dyes
4. Leibovitz's medium
5. 10p pipette/tips
6. 200p pipette/tips

7. 1000p pipette/tips
8. Timer
9. Poly-L-Lysine coated chamber slides

III. Dye Preparation

1. Prepare stock solutions on the day of experiment

Stock preparation of **4 mM stock of Rhod-2, Molecular Probes, R1245**

- a. Molecular weight of Rhod-2 is 1123.96 and the vial contains 50 µg.
- b. Dissolve 50 µg of Rhod-2 (vial) in 10 µl DMSO to make up 5 mM Rhod-2
- c. Mix well by pipetting up and down.
- d. 0.3 µl of Rhod-2 stock needs to be added to 1 ml Leibovitz media to make a final concentration of 1.5 µM

Stock preparation of **2 mM stock of Fluo-4, Molecular Probes, F14201**

- a. Molecular weight of Fluo-4 is 1096.95 and the vial contains 50 µg.
- b. Dissolve 50 µg of Fluo-4 (vial) in 25 µl DMSO to make up 2 mM Fluo-4
- c. Mix well by pipetting up and down.

- d. 1.5 μ l of Fluo-4 stock needs to be added to 1 ml Leibovitz media to make a final concentration of 3 μ M

IV. T cell labeling Mitochondrial and Cytosolic Ca^{2+}

1. Labeling of Rhod-2, and Fluo-4 for mitochondrial and Cytosolic Ca^{2+}
 - a. Add 0.5 μ l of [3 mM stock] Fluo-4 solution into 1ml CD4+ T cell suspension.
 - b. Vortex for 2 sec.
 - c. Incubate at 37 deg.C. for 30 min.
 - d. Add 0.3 μ l of [5 mM stock] Rhod-2 solution into 1ml CD4+ T cell suspension.
 - e. Incubate at 37 deg.C. for 30 min.
 - f. Spin down at the speed 5 (Dr. Burghardt's centrifuge) for 5 min.
 - g. Resuspend cells in 1 ml Leibovitz's medium.
 - h. Incubate at 37 deg.C. for 15 min.
 - i. Spin down at the speed 5(Dr. Burghardt's centrifuge) for 5 min.
 - j. Resuspend cells in 1 ml Leibovitz's medium.
2. Mix cells (T cell:hybridoma = 5:1)
 - a. Mix 0.2 mL hybridoma into 1ml T cell suspension.
 - b. Transfer 1.2 mL of cell suspension from the tube to 1 well of the chambered slide.

- c. Visualize and see the best synapse. Capture images.
- d. Collect your images, report time after the experiment and bring to lab 323 in Kleberg. The images are analyzed using adobe photoshop on our 323 lab computer.

**A-17. Measurement of mitochondrial-to-cytosolic Ca^{2+} ratio by blocking
mitochondrial uniporter using RU-360**

**I. Preparation of poly-L-lysine (Sigma, cat#P8920)) coated chamber slides
(Lab-tech chambered cover glass slides, 31 German Borosilicate, Nunc, and
Cat #155380). 2 slides (4 chambers)**

1. Dilute 1ml poly-L-lysine in 9 ml sterile water.
2. Dispense 1.5 ml of diluted poly-L-lysine into each well of 2
slides (4 chambers)
3. Incubate for 30 minutes at RT.(leave in the hood and label: Do
not move)
4. Aspirate the solution and wash with 1 ml warm Leibovitz
before using

II. Isolate CD4+ T cells from spleens of WT/ *fat-1* mice

1. CD4+ T-cells at 2 million cells /ml/tube in RPMI media, incubate at 37°C
with 5% CO₂ (*Loosen the caps of the tubes when in the incubator)
2. Hybridoma (CD3+) at 2 million cells/ml – (*Cells in phenol free complete
DMEM media)
3. Mitochondrial dyes
4. Leibovitz's medium
5. 10p pipette/tips
6. 200p pipette/tips

7. 1000p pipette/tips
8. Timer
9. Poly-L-Lysine coated chamber slides

III. Dye Preparation

1. Prepare stock solutions on the day of experiment

Stock preparation of 4 mM stock of Rhod-2, Molecular Probes, R1245

- a. Molecular weight of Rhod-2 is 1123.96 and the vial contains 50 µg.
- b. Dissolve 50 µg of Rhod-2 (vial) in 10 µl DMSO to make up 5 mM Rhod-2
- c. Mix well by pipetting up and down.
- d. 0.3 µl of Rhod-2 stock needs to be added to 1 ml Leibovitz media to make a final concentration of 1.5 µM

Stock preparation of 2 mM stock of Fluo-4, Molecular Probes, F14201

- a. Molecular weight of Fluo-4 is 1096.95 and the vial contains 50 µg.
- b. Dissolve 50 µg of Fluo-4 (vial) in 25 µl DMSO to make up 2 mM Fluo-4
- c. Mix well by pipetting up and down.

- a. μl of Fluo-4 stock needs to be added to 1 ml Leibovitz media to make a final concentration of $3\text{ }\mu\text{M}$

Stock preparation of 10mM stock of RU-360, Calbiochem, 557440

- a. Take $\sim 5\text{ ml}$ nanopure water in a 15 ml falcon and blow nitrogen from the nitrogen tank using a clean new glass pipette for 10 min.
- b. Flush the lid with Nitrogen and tightly close the falcon tube and rush to the hood of cell culture and add the water into the vial of RU-360 and mix well
- c. Molecular weight of RU-360 is 550.8 and the vial contains $100\text{ }\mu\text{g}$.
- d. Dissolve $100\text{ }\mu\text{g}$ of RU-360 (vial) in $181.6\text{ }\mu\text{l}$ degassed nanopure water to make up 10 mM RU-360
- e. Mix well by pipetting up and down gently without introducing any air bubbles.
- f. $1.0\text{ }\mu\text{l}$ of RU-360 stock needs to be added to 1 ml Leibovitz media to make a final concentration of $10\text{ }\mu\text{M}$

IV. T cell labeling Mitochondrial and Cytosolic Ca^{2+} and mitochondria uniporter inhibitor

- 1. Incubation with RU-360 and labeling of Rhod-2, and Fluo-4 for mitochondrial and Cytosolic Ca^{2+}

- a. Add 1.0 ul of [10 mM stock] RU-360 solution into 1ml CD4⁺ T cell suspension.
- b. Vortex for 2 sec.
- c. Incubate at 37 deg.C. for 30 min.
- d. Spin down at the speed 5 (Dr. Burghardt's centrifuge) for 5 min.
- e. Resuspend cells in 1 ml Leibovitz's medium.
- f. Add 0.5 ul of [3 mM stock] Fluo-4 solution into 1ml CD4⁺ T cell suspension.
- g. Vortex for 2 sec.
- h. Incubate at 37 deg.C. for 30 min.
- i. Add 0.3 ul of [5 mM stock] Rhod-2 solution into 1ml CD4⁺ T cell suspension.
- j. Incubate at 37 deg.C. for 30 min.
- k. Spin down at the speed 5 (Dr. Burghardt's centrifuge) for 5 min.
- l. Resuspend cells in 1 ml Leibovitz's medium.
- m. Incubate at 37 deg.C. for 15 min.
- n. Spin down at the speed 5(Dr. Burghardt's centrifuge) for 5 min.
- o. Resuspend cells in 1 ml Leibovitz's medium.

2. Mix cells (T cell:hybridoma = 5:1)
 - a. Mix 0.2 mL hybridoma into 1ml T cell suspension.
 - b. Transfer 1.2 mL of cell suspension from the tube to 1 well of the chambered slide.
 - c. Visualize and see the best synapse. Capture images.
 - d. Collect your images, report time after the experiment and bring to lab 323 in Kleberg. The images are analyzed using adobe photoshop on our 323 lab computer.

A-18. Measurement of mitochondrial-to-cytosolic Ca^{2+} ratio by blocking CRAC channel using BTP-2

I. Preparation of poly-L-lysine (Sigma, cat#P8920)) coated chamber slides (Lab-tech chambered cover glass slides, 31 German Borosilicate, Nunc, and Cat #155380). 2 slides (4 chambers)

1. Dilute 1ml poly-L-lysine in 9 ml sterile water.
2. Dispense 1.5 ml of diluted poly-L-lysine into each well of 2 slides (4 chambers)
3. Incubate for 30 minutes at RT.(leave in the hood and label: Do not move)
4. Aspirate the solution and wash with 1 ml warm Leibovitz before using

II. Isolate CD4+ T cells from spleens of WT/ *fat-1* mice

1. CD4+ T-cells at 2 million cells /ml/tube in RPMI media, incubate at 37°C with 5% CO₂ (*Loosen the caps of the tubes when in the incubator)
2. Hybridoma (CD3+) at 2 million cells/ml – (*Cells in phenol free complete DMEM media)
3. Mitochondrial dyes
4. Leibovitz's medium
5. 10p pipette/tips
6. 200p pipette/tips
7. 1000p pipette/tips
8. Timer

9. Poly-L-Lysine coated chamber slides

III. Dye Preparation

1. Prepare stock solutions on the day of experiment

Stock preparation of 4 mM stock of Rhod-2, Molecular Probes, R1245

- a. Molecular weight of Rhod-2 is 1123.96 and the vial contains 50 μg .
- b. Dissolve 50 μg of Rhod-2 (vial) in 10 μl DMSO to make up 5 mM Rhod-2
- c. Mix well by pipetting up and down.
- d. 0.3 μl of Rhod-2 stock needs to be added to 1 ml Leibovitz media to make a final concentration of 1.5 μM

Stock preparation of 2 mM stock of Fluo-4, Molecular Probes, F14201

- a. Molecular weight of Fluo-4 is 1096.95 and the vial contains 50 μg .
- b. Dissolve 50 μg of Fluo-4 (vial) in 25 μl DMSO to make up 2 mM Fluo-4
- c. Mix well by pipetting up and down.
- d. 1.5 μl of Fluo-4 stock needs to be added to 1 ml Leibovitz media to make a final concentration of 3 μM

Stock preparation of 100 μ M stock of BTP-2, Calbiochem, 203890

- a. Molecular weight of BTP-2 is 421.3 and the vial contains 5mg.
- b. Dissolve 5 mg of BTP-2 (vial) in 1.1868 μ l of DMSO to make up 10 mM BTP-2. Take 10 μ l of 10 mM BTP-2 and add 1mL of DMSO to make 100 μ M of BTP-2 working stock.
- c. Mix well by pipetting up and down gently without introducing any air bubbles.
- d. 1 μ l of BTP-2 working stock needs to be added to 1 ml Leibovitz media to make a final concentration of 10 μ M

IV. T cell labeling Mitochondrial and Cytosolic Ca²⁺ and CRAC channel inhibitor

1. Incubation with BTP-2 and labeling of Rhod-2, and Fluo-4 for mitochondrial and Cytosolic Ca²⁺
 - a. Add 1.0 μ l of [100 μ M stock] BTP-2 solution into 1ml CD4⁺ T cell suspension.
 - b. Vortex for 2 sec.
 - c. Incubate at 37 deg.C. for 1 hr.
 - d. Spin down at the speed 5 (Dr. Burghardt's centrifuge) for 5 min.
 - e. Resuspend cells in 1 ml Leibovitz's medium.

- f. Add 0.5 ul of [3 mM stock] Fluo-4 solution into 1ml CD4+ T cell suspension.
 - g. Vortex for 2 sec.
 - h. Incubate at 37 deg.C. for 30 min.
 - i. Add 0.3 ul of [5 mM stock] Rhod-2 solution into 1ml CD4+ T cell suspension.
 - j. Incubate at 37 deg.C. for 30 min.
 - k. Spin down at the speed 5 (Dr. Burghardt's centrifuge) for 5 min.
 - l. Resuspend cells in 1 ml Leibovitz's medium.
 - m. Incubate at 37 deg.C. for 15 min.
 - n. Spin down at the speed 5(Dr. Burghardt's centrifuge) for 5 min.
 - o. Resuspend cells in 1 ml Leibovitz's medium.
2. Mix cells (T cell:hybridoma = 5:1)
- a. Mix 0.2 mL hybridoma into 1ml T cell suspension.
 - b. Transfer 1.2 mL of cell suspension from the tube to 1 well of the chambered slide.
 - c. Visualize and see the best synapse. Capture images.
 - d. Collect your images, report time after the experiment and bring to lab 323 in Kleberg. The images are analyzed using adobe photoshop on our 323 lab computer.

A-19. Calculation of Intensity at Region of Interest

1. Capture images and convert the image files to 8-bit TIFF format, using save as option on the zeiss image viewer software.
2. Open the image file in Adobe Photoshop® CS (v.8.0).
3. Using a marquee tool, make a circle to select cell-cell contact region.
4. In “Histogram” panel, we can choose each channel of RGB.
5. Once blue channel is selected, the panel displays “mean intensity” of marquee-selected region.
6. Histogram also shows the pixels chosen by marquee tool. We generally choose 800-1200 pixels for contact region.
7. If you change the channel to green, mean intensity is changed, while pixel is not.
8. For non-contact control cells, select whole cell by marquee tool.
9. Histogram shows green/blue mean intensities.
10. Record the intensity for green and red channel.

VITA

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Publications	<p>R.Yog, R.Barhoumi, David N.McMurray, R.S.Chapkin (2010) n-3Polyunsaturated fatty acids suppress mitochondrial translocation to the immunological synapse and modulate calcium signaling in T cells from <i>fat-1</i> mice. J.Immuno.184: 5865-5873.</p> <p>Sarkar S., C. Sharma, R. Yog, A Periakaruppsm, O. Jejelowo, R. Thomas, E. V.Barrera, A. C. Rice-Ficht, B.L.Wilson and G.T. Ramesh (2007) Analysis of stress responsive genes induced by single-walled carbon nanotubes in BJ foreskin cells. J.Nanosci. Nanotechnol.7: 584-592.</p>